# PHARMACOLOGICAL STUDIES OF BRADYKININ AND OTHER INFLAMMATORY MEDIATORS IN RAT NEURAL PREPARATIONS.

By

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**To my mum and dad**

## DECLARATION

I declare that this thesis was composed entirely by me and represents all my own work except for the procedures listed below.

1. Approximately 30% of the electrophysiological experiments studying the effects of bradykinin, adenosine, purines, adrenoceptor agents and indomethacin *in-vivo* were carried out in collaboration with Dr D.S. McQueen, Mr D. Kelly, Mr C. Marr, and Dr. M. Tazaki. In such cases either surgery or injection of drugs was carried out by these persons.

2. In approximately 65% of the behavioural studies using arthritic rats, the various parameters were measured by Mrs S. Bond and Mrs. A. Paterson.

A.U.R. Asghar

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## ABSTRACT

This thesis tests the hypothesis that certain mediators (purines, catecholamines and bradykinin), which are known to be involved in inflammation, contribute to the sensitisation of peripheral nociceptors that occurs in chronic inflammation and hyperalgesia associated with arthritis. The major part of the studies determined the role of these substances and their pharmacological receptors in modulating discharge recorded from high threshold C-fibre mechanonociceptors (on-going or 'spontaneous', and mechanically-evoked) in normal ankle joints and in those with a monoarthritis induced by Freunds complete adjuvant (FCA). Some of the *in-vivo* neuropharmacological investigations were complemented by behavioural studies on intact normal and arthritic rats. Related *in-vitro* experiments were performed involving a) extracellular 'grease gap' recordings from various peripheral nerves and b) contractile responses of the electrically-stimulated rat vas deferens.

Recordings from C-fibres in arthritic joints revealed an enhanced resting discharge, a greater number of receptive fields and lower mechanical activation thresholds (sensitisation) of mechanonociceptors as compared to untreated joints. Indomethacin significantly attenuated, but did not abolish, either the elevated resting discharges recorded from C-fibres in arthritic joints or the swelling, mechanical hyperalgesia and inflammation associated with functional studies in monoarthritic rats. These results with indomethacin indicate that although prostanoids are important mediators in FCA-

induced arthritis, other mediators are also likely to be responsible for the observed neuropharmacological and behavioural changes seen in this model of arthritis.

Close intra-arterial injection of adenosine $A_1$ and $A_2$ receptor agonists and antagonists had no effect on either spontaneous or mechanically-evoked discharge recorded from articular C-fibres in either normal or arthritic joints. Preliminary studies provided no evidence for the involvement of $P_2$ purinoceptors in modulating afferent articular neural discharge from either normal or arthritic joints.

Selective $\beta_2$-adrenoceptor agonists (salbutamol and salmeterol) and antagonists (ICI 118551) did not affect neural discharge recorded from mechanonociceptors in either normal or arthritic rat ankle joints. In line with these neuropharmacological experiments, functional studies involving chronic dosage treatment of animals with salmeterol or propranolol had no effect on the swelling, mechanical hyperalgesia or inflammation associated with arthritic rat ankle joints; dexamethasone significantly reduced joint swelling, inflammation and mechanical hyperalgesia.

Although adrenaline and noradrenaline did not affect neural discharge from mechanonociceptors in normal joints, these catecholamines did enhance both spontaneous and mechanically-evoked discharge in approximately 50% of mechanonociceptors recorded from arthritic joints. Since these effects were not affected by the $\beta$-adrenoceptor antagonist, propranolol, this suggests that the catecholamines were likely to be acting at $\alpha$-adrenoceptors. Further studies, which

were not possible in the present investigation, using selective α-adrenoceptor agonists and antagonists are needed to confirm this suggestion.

Close intra-arterial injection of bradykinin caused a dose-dependent increase in spontaneous and mechanically-evoked discharge recorded from mechanonociceptors both in normal and arthritic joints; responses to bradykinin were of lower magnitude in arthritic joints. The bradykinin $B_2$ receptor antagonist, Hoe140, differentially antagonised these responses to bradykinin: there was surmountable antagonism of bradykinin-evoked increase in the responsiveness to the standard mechanical stimulus, whereas there was insurmountable antagonism of bradykinin-induced excitation. These differential effects of Hoe140 may reflect subtypes of the bradykinin $B_2$ receptor. Agonists and antagonists selective for the bradykinin $B_1$ receptor had no effect on afferent neural discharge (spontaneous or mechanically-evoked) from mechanonociceptors in either normal or arthritic joints. A more detailed study of bradykinin and Hoe 140 was performed *in-vitro*. Changes in membrane potential were recorded from various isolated nerves (vagus, tibial, tibialis, sciatic and saphenous) using the 'grease-gap' technique. However, in this model bradykinin had no effect on the membrane potential of any of the nerves examined, so this technique was abandoned. An alternative preparation, the electrically-stimulated rat vas deferens, was used and responded to bradykinin with two distinct effects; an enhancement in the electrically-evoked twitch (neurogenic response) and an increase in the baseline muscle tension (musculotropic response). Hoe140 differentially antagonised these responses to bradykinin; there was surmountable antagonism of the bradykinin-

induced neurogenic response, but insurmountable antagonism of the musculotropic response. In addition, certain $B_2$ receptor agonists and antagonists had differential actions on the neurogenic and musculotropic responses, indicating that subtypes of the $B_2$ receptor may be present. Bradykinin $B_1$ receptor agonists and antagonists were ineffective in the electrically-stimulated rat vas deferens.

In conclusion, the results show that C-fibre afferent discharge from articular mechanonociceptors in the normal and chronically-inflamed (adjuvant-arthritic) rat ankle joint is modulated by bradykinin (excitation and sensitisation). Catecholamines can also cause excitation and sensitisation of arthritic joints, but purines appear to have no effect on the activity of these sensory receptors. Bradykinin, acting via bradykinin $B_2$ receptors plays an important role in altering neural excitability in the rat, and different subtypes of $B_2$ receptor may be involved. Overall, the present results add further to our knowledge and understanding of the peripheral mechanisms involved in nociception in the normal state and in chronic inflammation.

## PUBLICATIONS

Asghar, A.U.R., McQueen, D.S. & Macdonald, A.E. (1992). Absence of effect of adenosine on the discharge of articular mechanoreceptors in normal and arthritic rats. *Br. J. Pharmacol.*, **105**. 309P.

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Asghar, A.U.R., McQueen, D.S., Marr, C.G. & Milne, SA. (1993). Characterisation of bradykinin receptors affecting discharge from articular sensory receptors in arthritic rats. 5th INWIN World conference, abstract nos, **283**.

Asghar, A.U.R., Wheeldon, A., Birch, P.J., Coleman, R.A. & McQueen, D.S. (1994). Pharmacological profiles of bradykinin analogues in the electrically-stimulated rat vas deferens. *Br. J. Pharmacol.,* Submitted.

## LIST OF COMMONLY USED ABBREVIATIONS

| | |
|---|---|
| ANOVA | analysis of variance |
| ATP | adenosine triphosphate |
| BK | bradykinin |
| $Ca^{2+}$ | calcium cation |
| °C | degrees Celsius |
| cAMP | cyclic adenosine monophosphate |
| $CO_2$ | carbon dioxide |
| d | day |
| g | gram |
| hr | hour |
| $H^+$ | hydrogen ion |
| 5-HT | 5-hydroxytryptamine |
| i.a. | intra-arterial |
| i.p. | intra-peritoneal |
| i.p.s. | impulses per second |
| $K^+$ | potassium ion |
| kg | kilogram |
| log | logarithm |
| M | molar concentration |
| m | metre |
| m (prefix) | milli ($10^{-3}$) |

| | |
|---|---|
| mg | milligram |
| $mgkg^{-1}$ | milligrams per kilogram |
| $ms^{-1}$ | metres per second |
| µg | microgram |
| µV | microvolts |
| min | minute |
| ml | millilitre |
| mm | millimetre |
| n | number of observations |
| $Na^+$ | sodium ion |
| NaCl | sodium chloride |
| NSAID | non-steroidal anti-inflammatory drug |
| $O_2$ | oxygen |
| P | statistical probability |
| s | second |
| s.e.m. | standard error of the mean |
| w/v | weight per volume |

Other abbreviations, are defined at the first point of occurrence in the text.

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*SECTION 1*

# *INTRODUCTION*

# INTRODUCTION

## 1.1 Pain, nociception and sensitisation

Early observations from the work of Blix (1884) and Von Frey (1895) provided evidence for the existence of specialised sensory organs which were responsible for the signalling of noxious stimuli. These specialised sensory organs were termed as nociceptors by Sherrington (1906). Under normal physiological conditions, intense or potentially damaging noxious stimuli activate nociceptors ('pain receptors' / high threshold sensory receptors) with the resulting pain being highly localised in nature and, if no tissue damage has occurred, usually of short duration (see Woolf, 1991). The function of this physiological pain is to inform of and protect the body from potential injury, which is why it is highly correlated with the initiation of the flexion withdrawal reflex (Willer 1979; Woolf, 1991). The amplitude of physiological pain is closely related to the intensity of the stimulus and has distinct mechanical, thermal and chemical activation thresholds. Thus, physiological pain can be considered to involve nociceptor-mediated pain. In contrast, pathological pain can arise following tissue damage and inflammation, and under this circumstance pain can occur in the absence of a clear stimulus, in response to innocuous stimuli, and in an enhanced and prolonged fashion following noxious stimuli. These features, which represent an increase in the sensitivity of the nociceptive sensory system, have been termed as sensitisation. Such sensitisation results from changes in the properties of peripheral terminals of primary afferent nociceptors (peripheral sensitisation) and from changes in the properties of spinal cord or supraspinal neurones (central sensitisation).

Although pathological or inflammatory pain in its acute form has a physiological role (contact to stimuli, whether noxious or innocuous, is avoided thus preventing any further damage and allowing tissue repair), in its chronic form it no longer has an adaptive function. In chronic pain there appears to be little correlation with the amplitude of a given stimulus, and indeed pain can outlast the original tissue inflammation (Woolf, 1991).

It is thus clear that, if new drugs are to be developed to treat the pain associated with chronic inflammatory conditions such as rheumatoid arthritis, it is necessary to obtain a detailed understanding of the changes in sensory receptor sensitivity that take place during inflammation.

## 1.2 Articular sensory receptors

Although there is a large body of work which has investigated sensory receptors in many tissues (particularly skin), only joint sensory receptors are described here because of their particular relevance to the present experimental work.

### 1.2.1 Anatomy and histology of articular sensory receptors

Joints are supplied by articular branches descending from main nerve trunks or by their muscular, cutaneous and periosteal branches (see Polacek, 1966). Articular nerves contain myelinated and unmyelinated sensory afferent fibres and unmyelinated efferent sympathetic postganglionic fibres (Skoglund, 1956; Polacek, 1966; Freeman & Wyke, 1967; Langford & Schmidt, 1983; Guilbaud et al., 1985). Morphological

studies in animals (Andrew & Dodt, 1953; Hromda & Polacek, 1958; Polacek,1966) have enabled the classification of articular sensory endings into four main types (Freeman & Wyke, 1967), namely:

1) Ruffini-type corpuscles: these are clusters (3 - 6) of encapsulated nerve endings emanating from one small-diameter myelinated axon. This type of nerve ending is found predominantly in the fibrous layer of the joint capsule, although a small number are located on extrinsic ligaments, para-articular periosteum, and related tendons.

2) Pacinian-type corpuscles: these are of elongated cylindrical shape (thick laminated capsule) and are found individually or in clusters of 2 - 3 and are innervated by branches from a single myelinated axon. They are found mostly in the deeper capsular tissues with a small number in the articular fat pads (where attached to joint capsule). They are more likely to be found on the lateral aspect of the joint.

3) Golgi-tendon (fusiform) corpuscles: these have a structure which is similar to the Ruffini-type corpuscle. They are innervated by a single large myelinated axon and are found only in joint ligaments and tendons.

4) Free nerve endings: these are non-corpuscular terminations composed of unmyelinated nerve filaments and are associated with unmyelinated or finely myelinated axons. Sensory axons consist of a series of spindle-shaped 'beads' which are connected by thinner segments without Schwann cell processes (Heppelmann et

al., 1990). These beads and the end bulb contain numerous vesicles, glycogen particles and mitochondria and, are assumed to represent multiple receptive sites (Heppelmann et al., 1990). Free nerve endings provide the majority of joint afferent innervation and are found distributed throughout the fibrous capsule, adjacent periosteum, articular fat pads, tendons, ligaments and in the advential sheaths of articular blood vessels. There is also evidence that free nerve endings are found in the synovial membrane (Halata & Groth, 1976; Halata et al., 1984).

Although the above classification of articular sensory receptors is from cats and rats, human joints also appear to contain similar articular sensory receptors (Schultz et al., 1984; Schutte et al., 1987; Zimny, 1988; De Avila et al., 1989).

### 1.2.2 Electrophysiological studies of joint sensory receptors

Electrophysiological studies, mainly in rats, cats and monkeys, were initially focused on examining the response properties of those receptors (i.e. Ruffini-, Pacinian- and Golgi-type) that are associated with myelinated fast-conducting afferent fibres (see review by Schaible & Grubb, 1993). Ruffini corpuscles are excited by flexion, extension, and the application of direct pressure (low mechanical activation threshold). These endings show a slowly adapting discharge in response to maintained stretch of the capsule (Boyd, 1954; Eklund & Skoglund, 1960; Burgess & Clark, 1969; Grigg & Hoffman, 1982). Pacinian corpuscles show a rapidly adapting response to low threshold stimulation (Burgess & Clark, 1969). Sensory endings of the Golgi-

type are excited by flexion or the application of light pressure to ligaments and show a slowly-adapting response (Burgess & Clark, 1969; Grigg & Hoffman, 1982).

In more recent years electrophysiological recordings have been made from unmyelinated or finely myelinated afferent articular fibres that are associated with free nerve endings (see review by Schaible & Grubb, 1993). Unmyelinated afferent fibres (also termed C- or Group IV fibre afferents) have conduction velocities $< 2.5 ms^{-1}$, whereas thin myelinated afferent fibres (termed Aδ or Group III afferents) have conduction velocities of between 2.5 - $20 ms^{-1}$ (see Schaible & Schmidt, 1983a,b; Heppelmann et al., 1988; Hildebrand et al., 1991; Schaible & Grubb, 1993). Various investigations demonstrated that C- or Aδ-fibre afferent units were only activated by joint movements outside the normal physiological range (e. g. extreme rotation), had high mechanical activation thresholds, and were excited by algogens (Coggeshall et al., 1983; Schaible & Schmidt, 1983a; Guilbaud & Iggo, 1985; Guilbaud et al., 1985; Kanaka et al., 1985; Grigg et al., 1986; Heppelmann et al., 1986; Russell et al., 1987; Neugebauer et al., 1989; He et al., 1990; Birrell et al., 1990; Grubb et al., 1991; Schepelmann et al., 1992; Schaible & Grubb, 1993). These observations suggested that C- and Aδ- fibres are associated with articular nociceptors which are likely to be involved in the signalling of joint pain. Furthermore, the sensitivity of articular nociceptors can be altered under inflammatory conditions. Thus, following acute inflammation of the knee joint, evoked by an intra-articular injection of kaolin and carrageenan, high-threshold afferents had reduced mechanical thresholds (sensitisation) such that they were now activated by innocuous flexion and extension

and some afferents developed on-going discharges (Coggeshall et al., 1983; Schaible & Schimdt, 1985; Grigg et al., 1986). A lowering of the mechanical activation threshold and the presence of on-going discharges has also been found for mechanonociceptors in chronically-inflamed (arthritic) rat ankle joints (Guilbaud et al., 1985; Grubb et al., 1991). The possible involvement of various inflammatory chemicals in mediating the altered sensitivity of nociceptors under inflammatory conditions is discussed below (see Section 1.3).

## 1.3 Inflammatory mediators in pain and nociception

The response to tissue injury is complex and involves the release of numerous chemicals that mediate and/or facilitate the inflammatory process. Inflammatory mediators originate from both non-neural and neural sources. The former includes various inflammatory cells such as mast cells, macrophages, lymphocytes and polymorphonuclear leukocytes, and the latter, somatosensory afferent and postganglionic sympathetic efferent nerve terminals (see Woolf, 1991).

A large number of endogenous mediators have been shown to have a role in the inflammatory process, including: prostanoids, bradykinin, purines, adrenergic agents, protons, 5-hydroxytryptamine, substance P, histamine & leukotrienes (see Higashi, 1986; Doherty & Wong, 1988; Rang et al., 1991; Schaible & Grubb, 1993; Dray et al., 1994). Furthermore, a number of putative endogenous inflammatory mediators such as nitric oxide, nerve growth factor and the cytokines are currently being evaluated (see Dray et al., 1994). One important factor in the study of inflammatory

mechanisms is that although inflammatory mediators may act independently, they are more likely to act synergistically (i.e. whereby the action of one mediator is dependent / enhanced by the action of another).

Two important effects of inflammatory mediators on sensory afferent neurones of particular relevance to the work presented in this thesis are excitation and sensitisation of nociceptors. Excitation is an induction or increase of afferent neural firing following the application of a stimulus (e.g. chemical, mechanical, thermal or electrical). Nociceptor sensistisation or hyperalgesia is characterised by 1) a decreased threshold for nociceptor activation and 2) an increased responsiveness to stimuli (e.g. chemical, mechanical, thermal or electrical). Inflammatory mediators vary in the extent to which they can cause excitation and/ or sensitisation of nociceptors. For example, bradykinin causes excitation, and increases responsiveness to mechanical stimuli, of high threshold mechanonociceptors in the rat ankle joint (Grubb et al., 1991). In contrast, the same study also showed that, although $PGE_2$ does not excite or cause sensitisation to mechanical stimuli, it does potentiate the excitatory action of bradykinin and enhances its sensitising effect on mechanical stimuli.

Although there are many inflammatory mediators that are involved, or are currently being investigated in chronic pain and hyperalgesia, the experimental work presented in the subsequent sections of this thesis examined only the roles of bradykinin, purines and adrenergic agents.

### 1.3.1 Bradykinin

#### 1.3.1.1 Biosynthesis and inactivation of bradykinin

Kinins are polypeptides that include bradykinin, lysine-bradykinin and methionine-lysine-bradykinin (see Figure 1.1 for amino acid sequences) which are generated in plasma or tissue following the breakdown of precursors called kininogens by the proteolytic action of plasma or tissue kallikrein (see Figure 1.2 and Marceau et al., 1980; Proud & Kaplan, 1988; Sharma, 1991).

The kininogens are multifunctional proteins that are mainly derived from $\alpha_2$-globulin having the kinin sequence in their molecules, and are synthesised in the liver and circulate in plasma and other body fluids (Sharma, 1991). In mammals three types of kininogens have been described ,namely: (i) a high molecular weight kininogen (HMWK) in the blood, (ii) a low molecular weight kininogen (LMWK) in both blood and body tissues, and (iii) an acute phase protein called T-kininogen that occurs only in the rat (Bhoola et al., 1992) - the mode of T-kininogen formation in the rat plasma and inflammatory fluid is summarised in Figure 1.3. In animals, increased levels of plasma kininogen have been reported following various inflammatory reactions, including adjuvant-induced arthritis (VanArman & Nuss, 1969; Borges & Gorden, 1976; Reis et al., 1982; Sharma, 1991). T-kininogen has been found to be raised in rats with adjuvant-induced arthritis (Greenbaum, 1984) or carrageenan-induced inflammation (Barlas et al., 1985) without any alterations in HMWK or LMWK concentrations.

(a) bradykinin

| | |
|---|---|
| *amino acids* | **Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg** |
| *position* | **1 2 3 4 5 6 7 8 9** |

(b) Lysine-bradykinin (Kallidin or Lys-bradykinin)

| | |
|---|---|
| *amino acids* | Lys-**Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg** |
| *position* | **1 2 3 4 5 6 7 8 9** |

(c) Methionine-Lysine-bradykinin (Met-Lys-bradykinin)

| | |
|---|---|
| *amino acids* | Met-Lys-**Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg** |
| *position* | **1 2 3 4 5 6 7 8 9** |

Figure 1.1 Amino acid sequence of (a) bradykinin, (b) Lys-bradykinin and (c) Met-Lys-bradykinin. Phe = Phenylalanine, Pro = Proline, Gly = Glycine, Ser = Serine, Arg = Arginine, Lys = lysine and Met = methionine.

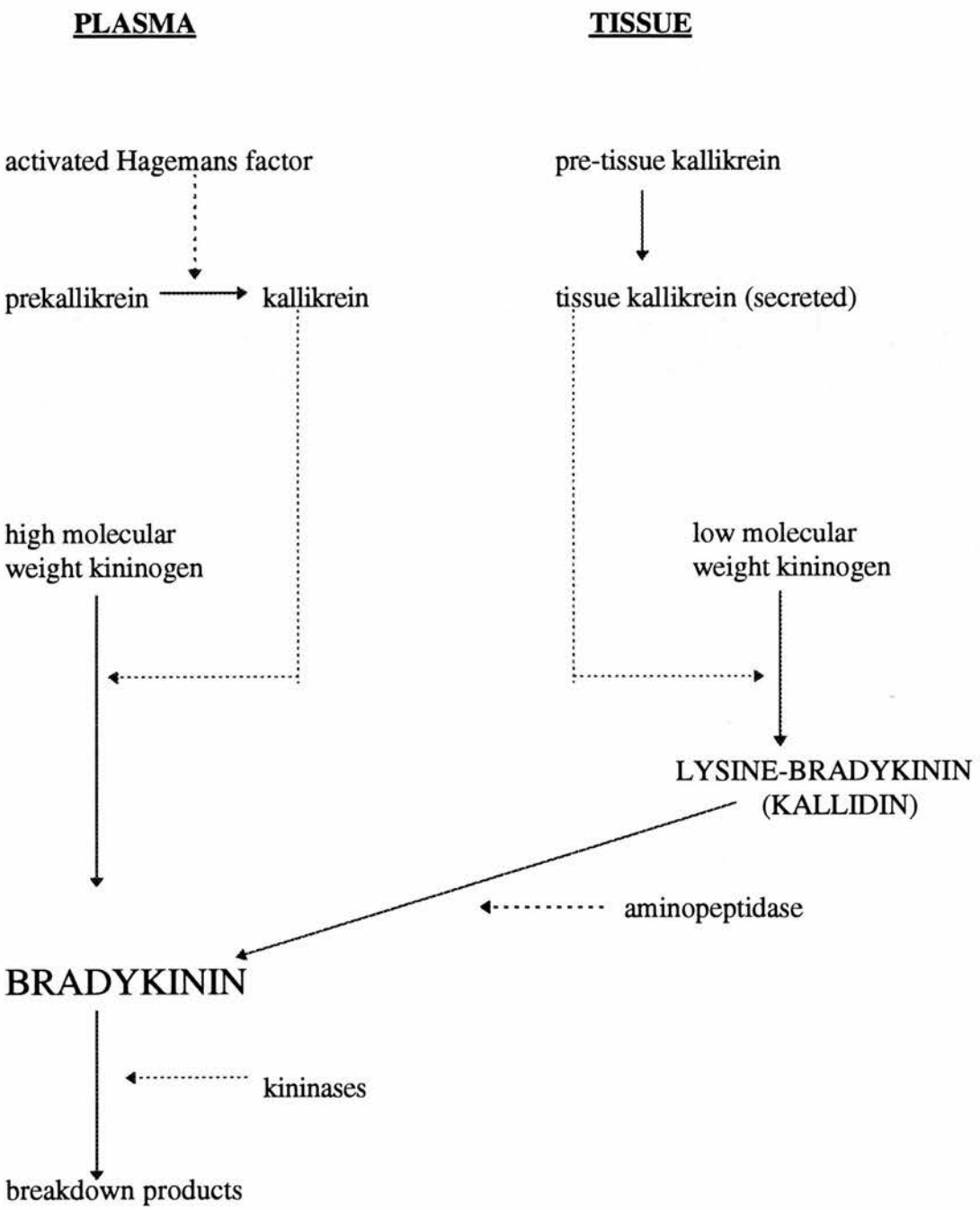



Figure 1.2 Schematic diagram showing the generation and breakdown of bradykinin. Solid arrows indicate transformations and dashed arrows indicate substances activating a transformation.

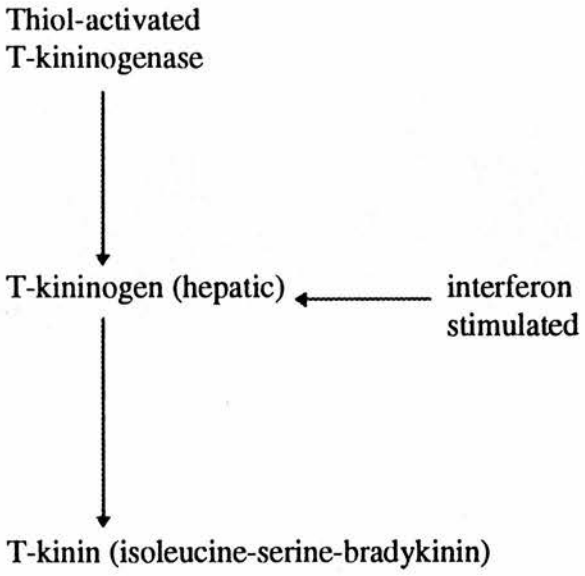



Figure 1.3 The mechanism of T-kinin formation in the rat.

Kallikreins are trypsin-like serine proteases which cause the breakdown of the kininogens to bradykinin or lysine-bradykinin (see Figure 1.2). There are two main kallikrein enzymes, namely plasma kallikrein and tissue kallikrein. These two kinin-forming enzymes differ from each other in molecular weight, action on kininogen substrates and the kinin formed (see Table 1.1 and Figure 1.2). The transformation of prekallikrein to kallikrein occurs in response to various activators ( e.g. Hagemans factor, nerve stimulation, plasmin, trypsin and immunoglobulin E) which arise as a result of tissue damage or inflammation. It has been demonstrated that synovial tissue kallikrein is increased in rheumatic patients as compared to healthy subjects (Sharma et al., 1983; Al-Haboubi et al., 1986).

Bradykinin and related kinins are inactivated by peptidases called kininases that are found in plasma, endothelial cells and tissues. There are two main types of kininases, termed Kininase I and Kininase II. Kininase I enzymes include plasma carboxypeptidase N and the cell membrane enzyme, carboxypeptidase M, both of which remove the C-terminal arginine from bradykinin to form des-Arg$^{9}$-BK. The Kininase II group of enzymes, angiotensin converting enzyme and neutral endopeptidase remove the two C-terminal amino acids (Phe-Arg) from bradykinin (Bhoola et al., 1992). Kininase II further degrades the remaining C-terminus by cleaving the dipeptide serine-proline. Although the concentration of kininase is always lower in synovial fluid as compared to blood, higher levels of kininase I are seen in rheumatoid or psoriatic arthritis (see Bhoola et al., 1992). Neutral endopeptidase has been indentified on the cell surface of human synovial cells, whereas no significant

**Table 1.1 Comparison of plasma and tissue kallikreins.**

| | **Plasma kallikrein** | **Tissue kallikein** |
|---|---|---|
| Approximate molecular weight | 85000 | 40000 |
| Plasma concentration ($\mu gml^{-1}$) | 50 | <1 |
| Kininogen substrate | high molecular weight kininogen | high and low molecular weight kininogen |
| Kinin formed | bradykinin | lysine-bradykinin |

angiotensin converting enzyme or carboxypeptidase N activities were found (see Bhoola et al., 1992). In joints, the degradation of bradykinin by synovial cells has been attributed entirely to neutral endopeptidase (see Bhoola et al., 1992).

#### 1.3.1.2 Bradykinin in pain and inflammation

Bradykinin is one of the most potent endogenously occurring algogenic agents and causes many pro-inflammatory effects including: vasodilation, tissue oedema and increased vascular permeability, release of histamine and eicosanoids, and stimulation of cell growth (for reviews see Marceau et al., 1983; Proud & Kaplan, 1988; Taylor et al., 1989; Bathon & Proud, 1991; Steranka & Burch, 1991; Bhoola et al., 1992). Endogenous bradykinin is implicated as an important inflammatory mediator in disorders such as rheumatoid arthritis, rhinitis, or asthma (see Proud & Kaplan, 1988; Bhoola et al., 1992). In man, bradykinin produces pain when applied to the cantharadin-induced blister base on the forearm (Keele & Armstrong, 1964; Whalley et al., 1987), or when it is injected intra-arterially (Coffman, 1966), intra-abdominally (Lim et al., 1967), sub-dermally (Ferreira, 1972), intra-muscularly (Jensen et al., 1990a) or intra-dermally (Jensen et al., 1990b). In animal studies, pseudoaffective responses (e.g. vocalisation, dextrorotation, biting, scratching, licking and reflex increase in blood pressure) indicative of pain are obtained following injection of bradykinin intra-arterially (Guzman et al., 1962; Guzman et al., 1964; Hashimoto et al., 1964), subdermally (Collier & Lee, 1963) or intra-articularly (Melmon et al., 1967; Moncada et al., 1975). In animal models of inflammation such as those induced by formalin (Corrêa & Calixto, 1993), carrageenan (Costello & Hargreaves, 1989),

collagenase (Legat et al., 1994) or *Porphyromonas gingivalis* (Griesbacher et al., 1994), endogenous bradykinin has been shown to be an important inflammatory mediator.

#### 1.3.1.3 Bradykinin and sensory afferent neural recordings

In electrophysiological recordings from C- or Aδ afferent fibres, bradykinin has been shown to excite nociceptors in skin (Fjallbrant & Iggo, 1961; Beck & Handwerker, 1974; Szolcsányi, 1987; Lang et al., 1990; Dray et al., 1992), viscera (Haupt et al., 1983; Mizumura et al., 1990;1992), skeletal muscle (Mense & Schimdt, 1974; Franz & Mense, 1975; Kumazawa & Mizumura, 1976; Mense, 1981;1982), trachea (Fox et al., 1993) and joints (Kanaka et al., 1985; Schepelmann et al., 1992). It has also been demonstrated that bradykinin can sensitise to mechanical (Mense & Meyer, 1987; Neugebauer et al., 1989; Grubb et al., 1991; Birrell et al., 1993) and thermal (Koltzenburg et al., 1992) stimuli. Evidence consistent with a role of bradykinin as a physiological mediator of pain has been provided by autoradiographic studies, where bradykinin receptors were shown to be localised to sensory neurons (Steranka et al., 1988).

Tachyphylaxis of bradykinin-induced increases in afferent neural discharge has been reported in many tissues such as the cat knee joint (Kanaka et al., 1985), dog testis (Kumazawa et al., 1987) and rat skin (Lang et al., 1990). In contrast, no tachyphylaxis to bradykinin was observed in afferents from dog viscera (Guzman et

al., 1962), cat muscle (Hiss & Mense, 1976; Mense et al., 1982) and cat cornea (Belmonte et al., 1994).

In general, the excitatory actions of bradykinin on nociceptors are found to be enhanced in the presence of other inflammatory mediators such as prostaglandins (Chahl & Iggo, 1977; Mense, 1981; Grubb et al., 1991; Birrell et al., 1993; Birrell & McQueen, 1993) or 5-HT (Mense, 1981). Consistent with this, bradykinin-induced excitation of muscle, visceral and cutaneous nociceptors were reduced by aspirin, paracetamol or indomethacin (Kumazawa & Mizumura, 1980; Mense, 1982; Dray et al., 1992). In contrast, it has been shown by Fox et al (1993) that bradykinin-evoked discharges, recorded from vagal afferents innervating the guinea-pig trachea, were not affected by ibuprofen, suggesting that bradykinin-induced responses were not dependent on, or influenced by endogenous prostaglandin production in this preparation.

#### 1.3.1.4 Bradykinin receptors

Bradykinin receptors were originally classified by Regoli & Barabé (1980) on the basis of relative potencies of agonists on isolated vascular smooth muscle preparations, and subdivided into two classes, $B_1$ and $B_2$. Thus, for example, $B_1$ receptors as found in rabbit aorta respond to kinin agonists with the following rank order of potency (see also Table 1.2):

des-Arg$^9$-bradykinin > Lys-bradykinin > bradykinin

**Table 1.2 Activities of kinins on the rabbit aorta ($B_1$ receptor) and jugular vein ($B_2$ receptor)**

| | Rabbit aorta | | Rabbit Jugular vein | |
|---|---|---|---|---|
| | **$pD_2$** | **R.A.** | **$pD_2$** | **R.A.** |
| Bradykinin | 6.22 | 100 | 8.48 | 100 |
| Lys-Bradykinin | 7.27 | 1122 | 8.63 | 141 |
| Des-Arg9-Bradykinin | 7.29 | 1175 | | <0.01 |

$pD_2$: negative log of the concentration that produces 50% of the maximum effect.
R.A.: Relative Affinity in percent of bradykinin. See also Regoli et al., 1990.

In contrast, $B_2$ receptors as found in rabbit jugular vein exhibit almost the opposite rank order of potency to kinin agonists (see also Table 1.2):

Lys-BK > bradykinin > des-Arg$^9$-bradykinin

On examination of the rank orders of potency from a vast number of different tissues, it is clear that bradykinin exhibits a much greater affinity for $B_2$ receptors than its carboxypeptidase N metabolite, des-Arg$^9$-bradykinin, and conversely, $B_1$ receptors are more sensitive to des-Arg$^9$-bradykinin than to bradykinin (Vavrek & Stewart, 1985; Taylor et al., 1989; Bathon & Proud, 1991; Rhaleb et al., 1991; Farmer & Burch, 1992). As a result, the relative potencies of des-Arg$^9$-bradykinin and bradykinin are used conventionally to classify a particular response as $B_1$ or $B_2$ mediated.

#### 1.3.1.4.1 Bradykinin $B_1$ receptors

In general, $B_1$ receptors have a limited distribution, with the main location studied being the rabbit aorta (Regoli et al., 1978; Bouthillier et al., 1987; DeBlois et al., 1988; Farmer et al., 1991a). Interestingly, in this tissue $B_1$ receptors are generated *de novo* in a time-dependent manner during *in-vitro* incubations, as shown by the increased responsiveness to the $B_1$ agonist, des-Arg$^9$-bradykinin (Bouthillier et al., 1987; DeBlois et al., 1988). Such an induction of $B_1$ receptors has also been demonstrated *in-vitro* in the mesenteric (Churchill & Ward, 1986; DeBlois & Marceau, 1987), coeliac (Ritter et al., 1989) and basilar (Whalley et al., 1983) arteries of the rabbit. Cytokines have been implicated in the induction of $B_1$ receptors. For

example, IL-1 is potent in its stimulation of responses to des-Arg$^9$-bradykinin in the rabbit aorta (see Burch & Kyle, 1992; Farmer & Burch, 1992). That IL-1 may be important in induction of aortic responses to des-Arg$^9$-bradykinin was evidenced by the observation that glucocorticoid treatment (inhibits IL-1 synthesis) inhibited the development of $B_1$ responsiveness (DeBlois et al., 1988).

The development of responsiveness to des-Arg$^9$-bradykinin during *in vitro* incubations has lead investigators to propose that noxious stimuli from tissue damage or inflammation may lead to $B_1$ receptor induction. In support of this concept are studies showing induced responses to des-Arg$^9$-bradykinin in rabbits with antigen-arthritis (Farmer et al., 1991a) or rabbits pretreated with either lipopolysaccharide (Regoli et al., 1981) or Triton X-100 (Marceau et al., 1980). Recently, the importance of $B_1$ receptors in chronic pain, hyperalgesia and inflammation has been demonstrated in rat models of persistent inflammatory thermal (Perkins & Kelly, 1993) or mechanical (Davis & Perkins, 1994) hyperalgesia. There is also evidence that $B_1$ receptors participate in both phases of the formalin-induced inflammation in the mouse (Corrêa & Calixto, 1993).

Bradykinin $B_1$ receptor antagonists arose from the observation that the agonist action of des-Arg$^9$-bradykinin is dependent upon the presence of phenylalanine at position 8 of the bradykinin sequence (Figure 1.1). Replacement of this phenylalanine with aliphatic amino acids was found to confer selective competitive $B_1$ receptor antagonist activity (Regoli et al., 1977). The most widely used potent, selective and

competitive $B_1$ receptor antagonists are des-Arg$^9$-Leu$^8$-bradykinin and its related analog, des-Arg$^{10}$-Leu$^8$-Lys-bradykinin (for review see Taylor et al., 1989; Bathon & Proud, 1991; Farmer & Burch, 1992). Recently, more potent and stable bradykinin $B_1$ receptor antagonists, such as des-Arg$^{10}$-Hoe140, have been developed (Wirth et al., 1991a).

The signal-transduction pathways activated by $B_1$ receptor stimulation are presently very poorly understood. Several workers have shown that des-Arg$^9$-bradykinin does not stimulate eicosanoid synthesis in rabbit smooth muscle (Regoli & Barabé, 1980), murine macrophages (Tiffany & Burch, 1989) or in human fibroblasts (Goldstein & Wall, 1984). In contrast, it has also been reported that $B_1$ receptor agonists do stimulate eicosanoid production in endothelial cells (Cahill et al., 1988; Conklin et al., 1988).

#### 1.3.1.4.2 Bradykinin $B_2$ receptors

In contrast to $B_1$ receptors (see Section 1.3.1.4.1), $B_2$ receptors show a widespread distribution being particularly evident in peripheral and central neurones, vascular smooth muscle, epithelium and various inflammatory cells (for review see Taylor et al., 1989; Bathon & Proud, 1991; Farmer & Burch, 1992). The vast majority of the effects of bradykinin such as pain, hyperalgesia, smooth muscle contraction or relaxation, increase in vascular permeability, reduction in blood pressure and release of inflammatory mediators are mediated by its activation of $B_2$ receptors (Taylor et

al., 1989; Bathon & Proud, 1991; Steranka & Burch, 1991; Bhoola et al., 1992; Burch & Kyle, 1992; Farmer & Burch, 1992).

The substitution of the proline residue at position 7 with D-phenylalanine in the bradykinin sequence (Figure 1.1) was perhaps the most important step in the development of bradykinin $B_2$ receptor antagonists (Vavrek & Stewart, 1985). Subsequently, a vast number of $B_2$ antagonists based on DPhe$^7$-bradykinin were synthesised and studied in a range of tissues (see Steranka et al., 1989; Burch et al., 1990; Regoli et al., 1990; Stewart & Vavrek, 1990; Rhaleb et al., 1991). Although these early bradykinin $B_2$ receptor antagonists made a remarkable contribution to the understanding of bradykinin-receptor pharmacology, they were of weak affinity and potency ($pK_B$ 5 - 7) and susceptibile to rapid enzymatic degradation (Griesbacher et al., 1989; Steranka et al., 1989; Burch et al., 1990). Recently, new bradykinin $B_2$ receptor antagonists, based on modified amino acids in positions 7 and 8 of bradykinin's primary sequence, have been synthesised and shown to be long-acting, with potencies two or three orders of magnitude greater than the earlier antagonists (Bao et al., 1991; Hock et al., 1991; Kyle et al., 1991; Lembeck et al., 1991; Burch & Kyle, 1992). D-Arg-[Hyp$^3$, Thi$^5$, D-Tic$^7$, Oic$^8$]-bradykinin (Hoe140) is one such potent $B_2$ receptor antagonist, and has recently been evaluated in various smooth muscle preparations (Hock et al., 1991; Lembeck et al., 1991; Field et al., 1992; Rhaleb et al., 1992; Griesbacher & Lembeck, 1992; Liebmann et al., 1993; Félétou et al., 1994) or in various models of acute and chronic inflammation (Wirth et al., 1991; Damas &

Remacle-Volon, 1992; Corrêa & Calixto, 1993; Heapy et al., 1993; Griesbacher et al., 1993; Griesbacher et al., 1994).

The observed differences in the responsiveness to a series of receptor agonists and antagonists is generally taken as evidence to suggest the existence of subclasses of receptor (Kenakin, 1984). Such a proposed subclassification is then, ideally, confirmed by radioligand binding studies, in which the potencies of ligands are correlated with their binding affinities (Weiland & Molinoff, 1981). There have been no studies in the bradykinin field to date which have rigorously satisfied all the criteria for subclassification of the bradykinin $B_2$ receptor. Nevertheless, evidence that there may be different subtypes of the $B_2$ receptor has been provided by various studies, such as those in rat myometrial membranes (Liebmann, 1991), rat uterus, guinea-pig ileum and trachea (Plevin, 1988), murine neuroblastoma cells (Braas et al., 1988) and in the electrically-stimulated rat vas deferens (Huidobro-Toro et al., 1986; Llona et al., 1987; Rifo et al., 1987).

Activation of $B_2$ receptors has been reported to cause stimulation of a variety of intracellular events. For example, phospholipase C is activated and causes the formation of inositol phosphates, which cause a rise in intracellular calcium and in diacylglycerol which activates protein kinase C (Higashida & Brown, 1988; Portilla et al., 1988; Chuang, 1989; Kaya et a., 1989; Perney & Miller, 1989; Burch et al., 1990). Protein kinase C has been shown to be an important second messenger in the action of bradykinin in various rat sensory neurons including skin C-fibre afferents

(Dray et al., 1988; Dray et. al., 1992) and dorsal root ganglion neurons (Burgess et al., 1989; Boland et al., 1991; McGuirk & Dolphin, 1992). An increase in conductance to $Na^+$ (depolarisation / excitation) is postulated to arise following protein kinase C activation by bradykinin (Burgess et al., 1989; Boland et al., 1991). This depolarisation caused by bradykinin appears to be sufficient to induce the opening of the high-threshold, dihydropyridine-sensitive, L-type calcium channels as bradykinin-induced influx of radiolabelled calcium is markedly reduced by nifedipine (Burgess et al., 1989; Boland et al., 1991). Activation of $B_2$ receptors has also been reported to cause stimulation of other intracellular events, including: stimulation of eicosanoid production probably as a result of phospholipase $A_2$ activation (see Bathon & Proud, 1991; Farmer & Burch 1992); accumulation of cyclic AMP (Smith et al., 1990; Farmer & Burch, 1992) and GMP (Schini et al., 1990) which is frequently due to an indirect stimulation of the biosynthesis of eicosanoids (Smith et al., 1990). Since bradykinin can cause inhibition of a slow after-hyperpolarisation, an effect mediated by cyclic-AMP, this may be one of the mechanisms underlying bradykinin-induced sensitisation (Weinreich,1986; Weinreich & Wonderlin, 1987).

Many studies have provided indirect evidence which suggests that $B_2$ receptors are coupled to G proteins (Higashida et al., 1986; Burch & Axelrod, 1987; Murayama & Ui, 1987). This hypothesis has been confirmed by the recent cloning of a $B_2$ receptor from the rat uterus (McEachern et al., 1991) and from a human lung fibroblast cell line (Hess et al., 1992), where the amino-acid sequences predict a protein which has characteristics of a seven transmembrane G protein-coupled receptor.

#### 1.3.1.4.3 Other bradykinin receptor subdivisions

Since bradykinin $B_1$ and $B_2$ receptor antagonists did not inhibit bradykinin binding, bradykinin-induced tracheal contractions or bradykinin-induced bronchoconstriction in the guinea-pig, this led to the proposed existence of the airway bradykinin $B_3$ receptor (Farmer et al., 1989; Farmer et al., 1991b; Farmer & DeSiato, 1994). Bradykinin $B_3$ receptors have also been proposed in the guinea-pig taenia caeci (Field et al., 1992). There is also evidence from studies on the opossum lower oesophageal sphincter muscle preparation, that bradykinin $B_4$ (mediate relaxtion) and $B_5$ (mediate contraction) receptors may exist (Saha et al., 1990;1991). All of these proposed new bradykinin receptor subdivisions will need confirmation using molecular cloning techniques and the development of selective and potent receptor antagonists.

### 1.3.2 Adenosine

#### 1.3.2.1 Release of adenosine

In hypoxic or ischaemic tissue, as found in inflammatory lesions, it has been reported that adenosine is generated in large amounts (Edlund et al., 1983; Church & Holgate, 1986; Fredholm & Sollevi, 1986). In human inflammatory conditions, such as asthma, the release of adenosine has been demonstrated following allergen challenge in asthmatics (Mann et al., 1983; Church & Holgate, 1986). This evoked release of adenosine originated from mast cells as well as other inflammatory cells in the airways following antigen challenge, and from muscle contraction or regional

hypoxia within the lungs (Mann et al., 1983; Church & Holgate, 1986). Adenosine release, evoked by an elevated potassium concentration, has been shown to originate from small diameter primary afferent neurons because it is reduced by pre-treating rats (neonatal or adult) with the selective C-fibre neurotoxin, capsaicin (Sweeney et al., 1989). Electrical stimulation of rabbit un-myelinated nerve fibres has been demonstrated to induce adenosine realease (Maire et al., 1984). Release of endogenous adenosine has been demonstrated from synaptosomes (prepared from spinal cord) in response to capsaicin or an elevated potassium concentration (Sweeney et al., 1987; Sweeney et al., 1989). Adenosine release evoked by elevated potassium or electrical stimulation has also been extensively reported in brain synaptosomes or slices (for review see Stone, 1981; Phillis & Wu, 1981).

#### 1.3.2.2 Adenosine in pain and inflammation

Observations by Bleehen & Keele (1977) have shown that pain results from the application of adenosine to a cantharadin-induced blister base on the human forearm. Pain can also be evoked in humans when adenosine is injected into the coronary (Crea et al., 1990; Lagerqvist et al., 1990a), brachial (Sylvén et al., 1988b) and iliac (Lagerqvist et al., 1990a) arteries, or when it is injected intravenously (Conradsson et al., 1987; Sylvén et al., 1988a; Crea et al., 1990; Lagerqvist et al., 1990b), or into the abdominal aorta (Lagerqvist et al., 1990a). In contrast, injection of adenosine into hand veins does not evoke pain from venous and paravascular nociceptors (Klement & Arndt, 1992).

Behavioural studies (paw withdrawal in response to increasing pressure) in rats have shown that intradermal injection of adenosine causes direct cutaneous hyperalgesia (Taiwo & Levine, 1990). Paradoxically, it has been reported that adenosine also has antinociceptive or anti-inflammatory actions in various animal models including carrageenan-induced pleurisy (Schrier et al., 1990; Lesch et al., 1991) or paw inflammation (Firestein et al., 1993) and tail flick (Holmgren et al, 1983) or hot plate (Holmgren et al., 1986) tests in rats, and acetylcholine-induced writhing (Herrick-Davis et al., 1989) or substance P or N-Methyl-D-aspartate-induced nociceptive behaviour (Delander & Wahl, 1988) in mice.

### 1.3.2.3. Adenosine and sensory afferents

Intra-arterial injection of adenosine into the brachial arteries of human volunteers has been suggested to cause activation of forearm afferent fibres, as determined indirectly by measuring reflex sympathetic stimulation (Costa & Biaggioni, 1993). In animal studies, adenosine has been reported to excite cat carotid body (McQueen & Ribeiro, 1981; Moteiro & Ribeiro, 1987), rat vagal pulmonary (Cherniack et al., 1987; Runold et al., 1987) and rat renal pelvis (Katholi et al., 1985) afferents. Adenosine has also been reported to evoke depolarisations in the isolated rat vagus, although such reductions in membrane potential were small (Trezise et al., 1993)

### 1.3.2.4 Adenosine receptors

Purine receptors are classified into $P_1$ (adenosine receptors) and $P_2$ (ATP receptors) subtypes. The role of $P_2$ purinoceptors in pain and inflammation is discussed later (see

Section 1.3.3). The first proposal that cell membrane adenosine receptors could be subdivided into $A_1$ and $A_2$ subtypes was made by Van Calker et al. (1979), based on the observation that adenosine could either inhibit (via the $A_1$ subtype) or stimulate (via the $A_2$ subtype) adenylate cyclase. There is increasing evidence that subtypes of adenosine $A_1$ ($A_{1a}$, $A_{1b}$, $A_{1c}$) and $A_2$ ($A_{2a}$, $A_{2b}$) receptors may exist, as well as an $A_3$ receptor (Linden, 1991; Collis & Hourani, 1993).

Selective adenosine $A_1$ or $A_2$ receptor agonists cause nociception or antinociception depending on which particular model of inflammation and route of administration is used. Thus, for example, intra-dermal injection of the selective adenosine $A_2$ receptor agonist, CV1808, causes direct cutaneous mechanical hyperalgesia in the rat (Taiwo & Levine, 1990) whereas, when $A_1$ and/or $A_2$ selective agonists are injected intracerebroventrically (Herrick-Davis et al., 1989) or intrathecally (Holmgren et al., 1986; Sawynok et al., 1986) antinociception (tail flick, hot plate or acetylcholine-induced writhing tests), rather than nociception, results. The nociceptive, inflammatory, or antinociceptive actions of adenosine and adenosine receptor agonists are blocked by nonselective (e.g. theophylline, 8-phenyltheophylline), $A_1$ selective (e.g. DPCPX) or $A_2$ selective (e.g. CP-66713) adenosine receptor antagonists (see Snyder, 1981; Daly, 1982; Linden, 1991; Collis & Hourani, 1993).

### 1.3.3 ATP

#### 1.3.3.1 ATP release

Adenosine 5`-triphosphate (ATP) is a ubiquitous component of cells that is present at millimolar levels (see Rang et al., 1991). Thus, tissue injury or damage is likely to release large amounts in the vicinity of sensory nerve endings (see Rang et al., 1991). ATP could be released from various sources including C-fibre terminals, sympathetic efferents, endothelial cells and platelets (see Sawynok & Sweeney, 1989; Hoyle & Burnstock, 1991). ATP has been shown to release mediators that are involved in inflammation such as nitric oxide from endothelial cells (see Hoyle & Burmnstock, 1991). In synaptosomal preparations of nerve terminals from the dorsal horn of the spinal cord, from rats and guinea-pigs, ATP is released in response to depolarising stimuli or by the sensory neurotoxin, capsaicin (White et al., 1985; Sweeney et al., 1989). ATP is also liberated from peripheral endings of un-myelinated sensory nerve fibres in the rabbit ear following antidromic electrical stimulation (Holton & Holton, 1954).

#### 1.3.3.2 ATP in pain and inflammation

In humans, application of ATP to the cantharadin-induced blister base in the forearm has been reported to cause pain (Bleehen & Keele, 1977). Although no pain is caused by the application of intact erythrocytes to the human blister base, pain is produced after lysis (see Keele et al., 1982). Such pain is partly due to the high concentration of ATP, although other adenosine phosphates such as adenosine monophosphate and

adenosine diphosphate are also likely to be involved (Keele et al., 1982). In animal studies, ATP and ATP analogues cause activation of cat or rat sensory neurons (Krishtal et al., 1988; Burnstock, 1990; Illes & Nörenberg, 1993). ATP has also been reported to cause depolarisation of the rat isolated vagus nerve (Trezise et al., 1993). ATP can also cause the induction / release of other inflammatory mediators such as prostanoids from various organs (e.g. lung, liver and kidney: see Needleman et al., 1974) or cytokines from macrophages (see Rang et al., 1991). These inflammtory mediators can in turn excite or sensitise sensory neurones (see Rang et al., 1991).

#### 1.3.3.3 ATP receptors

ATP interacts at purinoceptors of the $P_2$ subtype (Burnstock & Kennedy, 1985; Burnstock, 1990; Hoyle & Burnstock, 1991). The $P_2$ receptor has been subclassified into two subtypes, $P_{2x}$ and $P_{2y}$, on the basis of the effects of structural analogues of ATP using classical pharmacological methods (O'Conner et al., 1991; O'Conner, 1992; Illes & Nörenberg, 1993). In addition, there is also evidence for two further $P_2$ receptor subtypes, namely $P_{2t}$ and $P_{2z}$, which have been characterised in studies on platelets and mast cells, respectively (Gordon, 1986; Pintor et al., 1993).

Electrophysiological experiments using peripheral neurones (various sensory ganglia of rats and cats) indicate the presence of $P_{2y}$-like purinoceptors (see Illes & Nörenberg, 1993), although other investigators report a mixed population of $P_{2x}$ and $P_{2y}$ receptors in the isolated cervical vagus nerve (Trezise et al., 1993) or centrally in the locus coeruleus (Harms et al., 1992; Tschöpl et al., 1992).

#### 1.3.3.4 ATP receptor antagonists

The few purine $P_2$ receptor antagonists that are presently available all have problems of receptor selectivity. Nevertheless, suramin and arylazidoaminopropionyl-ATP are two non-selective purine $P_2$ receptor antagonists that have been used widely to antagonise the effects of ATP and ATP analogs in various neuronal and smooth muscle preparations (Sneddon & Burnstock, 1984; Dunn & Blakeley, 1988; Leff et al., 1990; Trezise et al., 1993).

Receptor desensitisation has also been used as a method for blocking purine receptors. Thus, for example, although the purine $P_{2x}$ receptor agonist, α,β-methylene-ATP, initially causes a response (smooth muscle contraction or depolarisation) there is full desensitisation upon subsequent exposures. Indeed, such a desensitisation by α,β-methylene-ATP is a recognised identification criterion for $P_{2x}$ receptors (Burnstock & Kennedy, 1985; O'Conner et al., 1990; O'Conner, 1992)

### 1.3.4. Adrenoceptors

#### 1.3.4.1. Inflammation and adrenoceptors

The observations of Wall & Gutnick (1974) that many unmyelinated and myelinated afferent fibres innervating a rat neuroma were excited by adrenaline or noradrenaline, raised the possibilitiy that damaged fibres expressed and/or up-regulated adrenoceptors or that they became fuctional. A great deal of subsequent work by

various workers has confirmed that an appreciable number of damaged fibres in a variety of species show sensitivity to noradrenaline or adrenaline (see below, and review by Devor, 1991).

Adrenoceptors are classifed into two main divisions namely, α- and β-adrenoceptors. α-adrenoceptors are further subclassified into $\alpha_1$ and $\alpha_2$-subtypes, and β-adrenoceptors into the $\beta_1$, $\beta_2$ and $\beta_3$ subtypes (see Bowman & Rand, 1980; Rang and Dale, 1987). Subdivisions of both $\alpha_1$ and $\alpha_2$-adrenoceptors also exist (see Watson & Girdlestone, 1994). Although noradrenaline and adrenaline act at both α- and β-adrenoceptors, noradrenaline has greater affinity (receptor specificity) for α-adrenoceptors ($\alpha_1$ and $\alpha_2$), whereas adrenaline has a greater affinity for β-adrenoceptors ($\beta_1$ and $\beta_2$) (see Bowman & Rand, 1980; Rang and Dale, 1987; Watson & Girdlestone, 1994).

#### 1.3.4.2 Adrenoceptors in pain and inflammation

Adrenoceptors have been implicated in chronic inflammatory conditions in man, such as reflex sympathetic dystrophy (Schott, 1986; Schwartzman & McLellan, 1987) and rheumatoid arthritis (Kaplan et al., 1980; Levine et al., 1986a; Hannington-Kiff, 1990). In these disorders a reduction in pain and inflammation can be achieved by the use of adrenergic neurone blocking drugs, such as guanethidine, which cause catecholamine depletion (Loh & Nathan, 1978; Bonica, 1979; Levine et al., 1986a; Hannington-Kiff, 1990).

A contribution of adrenoceptors, particularly $\beta_2$-adrenoceptors, has been suggested from the results of behavioural studies of chronic pain and inflammation associated with arthritis (see Fitzgerald, 1989). For example, treatment with the non-selective β-adrenoceptor antagonist, propranolol, was found to decrease the signs and symptoms of inflammation in patients with rheumatoid arthritis (Kaplan et al., 1980). Similarly, in rats with adjuvant-induced arthritis, treatment with propranolol was shown to reduce the severity of joint injury (Levine et al., 1988). Moreover, in this model of arthritis, $\alpha_1$, $\alpha_2$ or $\beta_1$-adrenoceptor antagonists were without effects, whereas the $\beta_2$-adrenoceptor antagonists, butoxamine or ICI 118551, were found to decrease the degree of joint severity (Levine et al., 1988). It has also been demonstrated that the $\beta_2$-adrenoceptor agonist, salbutamol, or the catecholamine, adrenaline (acting at $\beta_2$-adrenoceptors), exacerbate adjuvant-induced arthritis (Coderre et al., 1990; Coderre et al., 1991). In another model of inflammation, carrageenan-induced mechanical hyperalgesia, the β-adrenoceptor antagonist, propranolol, partially blocked the reduced nociceptive threshold to pressure on the hind paw of the rat (Nakamura & Ferreira, 1987). The α-adrenoceptor antagonist, phentolamine, had no effect in this model of inflammation. Paradoxically, in other studies, $\beta_2$-adrenoceptor agonists have been reported to have anti-inflammatory actions. For example, $\beta_2$-adrenoceptor agonists such as salbutamol and, in particular the longer-duration $\beta_2$-adrenoceptor agonist, salmeterol (Ball et al., 1991), are inhibitors of mediator (histamine, leukotrienes and prostaglandins) release (Butchers et al., 1979; Butchers et al., 1991), bradykinin-induced plasma protein extravasation (Whelan et al., 1993), vascular

permeability & granulocyte accumulation (Whelan & Johnson, 1992) and carrageenan-induced oedema (Green, 1972).

### 1.3.4.3. Effects of adrenaline and noradrenaline on afferent nerves

Electrophysiological recordings from C- or Aδ-fibres have shown that adrenaline and noradrenaline cause excitation only in inflamed tissues. For example, afferent neural recordings from C- fibres in rabbit ears have shown that, although noradrenaline did not excite C-polymodal nociceptors in undamaged ears, approximately 60% of C-fibre nociceptors were excited by this catecholamine in ears with damaged auricular nerves (Sato & Perl, 1991). This noradrenaline-induced excitation was blocked by $\alpha_2$ (predominantly) and $\alpha_1$-adrenoceptor antagonists; the effects of β-adrenoceptor antagonists were not examined. In chronically lesioned nerves (neuromas) in the cat, adrenaline was shown to excite 40% of unmyelinated skin afferents (Häbler et al., 1987), although which adrenoceptor mediated this action was not studied. In the investigation by Sanjue & Jun (1989), rat skin nociceptors showed no responses to noradrenaline, although when there was a sustained neural discharge, induced by a compound algogenic substance (mixture of 5-HT, histamine, KCl and HCl), noradrenaline enhanced the discharge in 7 of 9 afferents. The adrenoceptor responsible for this noradrenaline-induced excitation was not determined in this study. Studies of rat myelinated afferents ending in a neuroma also report an adrenaline-induced excitation (Devor & Jänig, 1981). This excitation was blocked by the α-adrenoceptor antagonist, phentolamine, but not by the β-adrenoceptor antagonist, propranolol (Devor & Jänig, 1981).

Noradrenaline-induced sensitisation of the response to mechanical stimulation has been reported in rat cutaneous mechanonociceptors (Gold et al., 1994). Since such mechanical sensitisations were blocked by yohimbine, this suggests the involvement of $\alpha_2$- adrenoceptors (Gold et al., 1994).

#### 1.3.4.4 Location of action of adrenaline and noradrenaline

Of the many possible sites at which adrenaline and noradrenaline could cause enhancement of neural discharge, two include the sympathetic efferent nervous system and the terminals of C-fibre afferents. Regarding the sympathetic efferent nervous system, various studies have suggested that there is an indirect coupling of sympathetic efferent and afferent fibres (see Fitzgerald, 1989; Gonzales et al., 1991; McMahon, 1991; Schaible & Grubb, 1993; Gold et al., 1994). In such a scenario, adrenaline or noradrenaline act on adrenoceptors located on sympathetic postganglionic terminals to cause the release of prostaglandins and/or leukotrienes which then act on afferent terminals to cause excitation or sensitisation (see Rang et al., 1991; McMahon, 1991). Indeed the long latency of the effects of adrenaline and noradrenaline reported in various studies would fit with such an indirect mechanism of action (see McMahon, 1991). Studies by Levine et al. (1986b) have shown that noradrenaline-induced hyperalgesia in inflamed (chloroform-induced) paws of rats is mediated through an interaction with terminals of sympathetic neurones rather than by activation of primary afferent nociceptors. Similarly, the terminals of sympathetic neurones have also been reported to be the site of action of adrenaline (adrenal

medulla-derived) in contributing to the severity of adjuvant-induced arthritis (Coderre et al., 1990).

Since adrenaline and noradrenaline only have an effect in inflamed tissues, it would have to be postulated that the sympathetic nervous system undergoes changes under inflammatory conditions, for example, by up-regulation and/or expression of adrenoceptors, or the coupling of adrenoceptors to different second messenger systems in the presence of a raised calcium concentration caused by inflammation (Gold et al., 1994). However, the role of the sympathetic nervous system in affecting afferent neural discharge has been questioned by Scott (1994), who presents powerful arguments that it is more likely to be visceral afferents (terminology also includes peripheral structures), which travel within autonomic nerves, which are the location of action of adrenergic agents. Another possible location of action for adrenaline or noradrenaline are the afferent nociceptive terminals, where it is suggested that there is an expression or up-regulation of α-adrenoceptors (Devor, 1991; McMahon, 1991), although this is not supported by the studies of Levine et al. (1986b; 1988).

## 1.4 Adjuvant-induced arthritis in the rat as a model of joint pain and inflammation

### 1.4.1. Aetiology and pathological features

Arthritis induced by Freunds adjuvant is a widely used and accepted model of arthritis in the rat, which is associated with chronic pain and inflammation, (see Billingham & Davies, 1979; Rainsford, 1982; Billingham, 1983; Colpaert, 1987). This

form of arthritis is induced by injecting Freunds complete adjuvant (FCA), a mixture of heat-killed mycobacteria (*Mycobacterium tuberculosum* or *Mycobacterium butyricum*) in mineral oil, into the tailbase or footpad. Other species of bacteria (Flax & Waxman, 1963; Paronetto, 1970), water soluble peptidoglycans (Kohashi et al., 1976; 1977) and muramyl dipeptide (Kohashi et al., 1980) have also been used to induce arthritis.

FCA-induced arthritis has an immunological origin, where an epitope contained on a mycobacterial heat shock protein is cross-reactive with a self-antigen in joint cartilage (Van Eden et al., 1985; 1989). This epitope is recognised by rat T-cells which then leads to a hypersensitivity reaction to the bacterial antigen and also to the cartilage proteoglycans (Van Eden et al., 1985).

Two phases of inflammation are produced by the injection of FCA into the foot pad or tail base. In the first, there is an acute local inflammatory reaction which develops within the first few hours after injection, but subsides after 3 - 5 days. There then follows the second phase, where there is polyarthritis, with lesions in the eyes, ears, nose, skin, tail or genitals as well as profound weight loss (Pearson & Wood, 1959; Ward & Jones, 1962; Pearson, 1963; Pearson & Wood, 1963; Billingham & Davies, 1979; Rainsford, 1982). The severest inflammation in polyarthritis occurs in the joints of the hind limbs, where there is bone destruction, damage to tendons and loss of cartilage.

The incidence, severity and consistent induction of FCA-induced polyarthritis is affected by several factors including the concentration of mycobacterium and its degree of dispersion (Ward & Jones, 1962; Newbould, 1963), the site of FCA injection (Winder et al., 1969; Perper et al., 1971; Koga et al., 1976), strain of rat used (Zideck & Perlik, 1971; Freeman & West, 1972; Eisen et al., 1973) and the addition of compounds to FCA so as to improve the incidence of disease (Whitehouse et al., 1974).

### 1.4.2 Adjuvant-induced arthritis as a model of chronic pain

In order to test whether or not the FCA-induced model of arthritis in the rat is associated with chronic pain, investigators have measured several behavioural parameters that are indicative of pain. Examples of such parameters include: reduction in body weight (Colpaert et al., 1982; Colpaert & Van Den Hoogen, 1983; Calvino et al., 1987), marked decrease in locomotor activity (Calvino et al., 1987), scratching behaviour (De Castro Costa et al., 1981), irritability (Reuler et al., 1980; Colpaert et al., 1982) and hyperventilation (Colpaert & Van Den Hoogen, 1983). Although parameters such as body weight, scratching or hyperventilation are not direct pain 'markers', the similarity of their time-courses with the arthritis process is, albeit indirectly, indicative of chronic pain in the FCA-induced arthritic model. Further support that arthritic rats are associated with chronic pain comes from studies which show that these animals preferentially consume drinking water that contains analgesic drugs (Colpaert et al., 1980), and from studies which show that analgesic drugs (e.g.

aspirin) can improve mobility (Larson & Arnt, 1985) and reduce scratching behaviour (De Castro Costa et al., 1987).

### 1.4.3 Adjuvant-induced arthritis and hyperalgesia

Several groups have measured the hyperalgesia associated with FCA-induced arthritic rats by a range of tests including the Randall-Selitto (paw withdrawal on the application of increasing pressure) test (Kayser & Guilbaud, 1981; Kayser & Guilbaud, 1983; Hara et al., 1984; Butler et al., 1985; Calvino & LeBars, 1986; Calvino et al., 1987), tail flick test (Colpaert, 1979; Yonehara et al., 1983), hot plate test (Hara et al., 1984), rotorod grip strength (Perrine & Takesue, 1968), compression with forceps (Hirose & Jyoyama, 1971), foot bend (extension or flexion) procedure (Kuzuna & Kawai, 1975; Winter et al., 1979; Capetola et al., 1980; Calvino & LeBars, 1986; Calvino et al., 1987) and transcutaneous electrical stimulation (Okuyama & Aihara, 1984).

### 1.4.4. Mechanonociceptor responsiveness in adjuvant-arthritis: central and peripheral considerations

Neurones at several sites in the central nervous system show marked changes in activity when there is inflammation, such as in the FCA-induced arthritic rat model. Thus, although spinal dorsal horn, thalamic and cortical neurones in the normal rat respond only to intense noxious mechanical stimuli from the periphery (e.g. in joints), they respond to mild mechanical stimuli in rats with FCA-induced arthritis (Gautron & Guilbaud, 1982; Menetrey & Besson, 1982; Lamour et al., 1983; Kayser & Guilbaud,

1984). In the rat superficial dorsal horn, a response is evoked by a stimulus such as light pressure on the normal ankle joint, whereas in striking contrast, it is easy to evoke a vigorous sustained discharge in the arthritic joint (Menetrey & Besson, 1982). More recently, it has been demonstrated by Grubb et al. (1993) that neurones of the superficial and deep dorsal horn and of the ventral horn show either an induction or an increase in ongoing discharges and a reduction in the mechanical threshold (mechanical stimuli applied to the ankle joint) in rats with a unilateral chronic inflammation induced by FCA. Following repetitive stimulation of C-fibres, spinal cord neurones are also involved in the 'wind-up' phenomenon (Davis & Lodge, 1987; Dickenson & Sullivan, 1990; Thompson et al., 1990; Schaible & Grubb, 1993); wind-up is a term which describes successive elevations in the magnitude of the responses of a neurone to a constant repeated stimulus (see Schaible & Grubb, 1993). The response properties of the spinal neurons during arthritis do not just reflect the afferent impulses from the joint on a spike for spike basis. Indeed there are at least three main components that may interact to determine the actual changes in response properties, namely: 1) peripheral component (alterations in afferent inflow), 2) spinal component (intraspinal modification) and, 3) supraspinal component (changes in descending inhibition). Thus it is clear that the spinal cord shows a great deal of plasticity following adjuvant-induced arthritis (see also Schaible & Grubb, 1993).

The alterations in the processing of nociceptive information during chronic inflammation may be due to several mechanisms including: alterations in synaptic mechanisms, or in the connectivity of neurones in the sensory pathway that lead to

their becoming increasingly easily excited by formely ineffective sensory inputs (see Iggo, 1988); changes in the sensitivity of sensory fibres that make them more effective central excitants or less effective inhibitors (see Iggo, 1988);
changes in the concentrations of a large number of various ions and mediators including: potassium (spinal cord: Heinemann et al., 1990), 5-HT (spinal cord, brainstem and forebrain: Weil-Fugazza et al., 1979,1980), glutamate (spinal cord: Sorkin et al., 1992), noradrenaline (spinal cord: Weil-Fugazza et al., 1986), cholecystokinin (spinal cord: Chery-Croze et al., 1985) and endogenous opioids (spinal cord: Hollt et al., 1987; Weihe et al., 1989).

As well as alterations in the central nervous system there are also changes in peripheral nociceptors following inflammation. For example, Guilbaud et al. (1985) have shown that increased responsiveness to mechanical stimuli given in the periphery following chronic inflammation may be a result of alterations in the sensitivity (e.g. induced by inflammatory mediators, see Section 1.4.6) of peripheral nociceptors. The electrophysiological recordings by Guilbaud et al. (1985) in the periphery, investigated the properties of high threshold mechanonociceptors (C-fibre afferents) in ankle joints of normal and FCA-induced polyarthritic rats. In arthritic joints, mechanonociceptors had lower mechanical activation thresholds, higher levels of spontaneous discharge and a larger number of mechanically-sensitive receptive fields (each corresponding to a single afferent unit) as compared to those from normal joints (Guilbaud et al., 1985). The sizes of individual receptive fields were similar in normal and arthritic joints. Histological studies of the articular nerve supply revealed no

difference in the size or number of axons found in normal and arthritic joints (Guilbaud et al., 1985). Thus, a change in the total number of afferent fibres cannot explain the alterations in the sensitivity of joint mechanonociceptors in arthritic joints.

### 1.4.5 Adjuvant-induced monoarthritis

Although FCA-induced polyarthritis has been used widely, and justifiably, in a large number of neurophysiological, biochemical and immunological studies, its systemic and widespread nature is unnecessary in the study of nociceptive mechanisms as it is difficult to attribute observed changes specifically to the arthritis process. There are also ethical considerations in the use of such severely affected animals. Therefore, it would be desirable both on scientific and ethical grounds if a less severe and more localised FCA-induced arthritis could be obtained and used as an alternative to FCA-induced polyarthritis for studying nociceptive processes. Recent studies have shown that a discrete monoarthritis is obtained in rats following the localised injection of FCA (100 - 150μg *Mycobacterium tuberculosum* or *Mycobacterium butyricum*) around (subdermally) the ankle joint or into the foot pad (Grubb et al., 1991; Butler 1992; Donaldson et al., 1993). Thus, interpretation of data from studies examining the role of various inflammatory mediators using the monoarthritic model will be made easier, since any observed changes are more likely to be attributed to the arthritis process *per se*.

### 1.4.6 Role of inflammatory mediators in adjuvant-induced arthritis

The sensitisation of nociceptors in FCA-induced arthritis has been shown to be partly attributable to the actions of prostanoids. For example, it has been demonstrated that intra-venous injection of lysine acetylsalicylate reduces the enhanced responsiveness of ventro-basal thalamic neurones in arthritic rats (Guilbaud, 1988). Electrophysiological recordings from C-fibre afferents in polyarthritic rats also suggest the important involvement of prostanoids. Thus, although lysine acetylsalicylate had no effect on the discharge from mechanonociceptors in normal rat ankle joints, it did reduce the elevated spontaneous discharge and the enhanced responsiveness to mechanical stimuli that are associated with polyarthritic rat ankle joints (Guilbaud & Iggo, 1985). In agreement with these results of Guilbaud & Iggo (1985) in polyarthritic rats, lysine acetylsalicylate or paracetamol-induced reductions in the elevated mechanonociceptor discharges have been reported in rats with a discrete monoarthritis (McQueen et al., 1991). Since aspirin or aspirin-like drugs only partly reduce the raised neural discharges seen in arthritic joints it is, therefore, likely that other inflammatory mediators (see Section 1.3) may also play a role in the inflammation-induced sensitisation process.

## 1.5 Aims of the present investigation

From the discussions above it is apparent that certain chemical mediators can modulate the responsiveness of peripheral sensory receptors in normal tissues and, in particular, tissues which are chronically inflamed. Although many studies have

demonstrated that prostanoids have a substantial role in altering nociceptor responsiveness in chronically inflamed tissue, as for example in the arthritic rat ankle joint (Guilbaud & Iggo, 1985; Birrell, 1990; McQueen et al., 1991), it is possible that other inflammatory mediators such as bradykinin (see Section 1.3.1), purines (see Section 1.3.2 and 1.3.3) and catecholamines (see Section 1.3.4) may also be involved.

The aims of the present investigation were:

1) To confirm and further characterise the use of a localised FCA-induced arthritis of the rat ankle joint in the study of chronic pain and inflammation.

2) To determine the role of bradykinin in affecting neural discharge (spontaneous and mechanically-evoked) from C-fibre afferent mechanonociceptors *in-vivo* in normal rat ankle joints, and in those with a localised FCA-induced arthritis. In order to further characterise the effects of bradykinin and its analogs on neural excitability, two *in-vitro* neural preparations were used. In one preparation, any change in membrane potential was recorded extracellularly from various sensory nerves (vagus, tibial, tibialis, sciatic and saphenous) using a 'grease-gap' technique. In the other preparation, neurally as well as non-neurally-mediated responses were studied in the electrically-stimulated rat vas deferens.

3) To determine the effects of indomethacin on the elevated spontaneous discharge and the enhanced responsiveness to mechanical stimuli associated with C-fibre

afferent mechanonociceptors in rat ankle joints with a localised FCA-induced arthritis. Complementary behavioural studies were also performed in order to determine the effects of indomethacin in monoarthritic rats.

4) To determine the role of purines on neural discharge (spontaneous and mechanically-evoked) from C-fibre afferent mechanonociceptors *in-vivo* in normal rat ankle joints and in those with a localised FCA-induced arthritis

5) To determine the role of adrenoceptors, particularly the $\beta_2$-subtype, in modulating neural discharge (spontaneous and mechanically-evoked) from C-fibre afferent mechanonociceptors *in-vivo* in normal rat ankle joints and in those with a localised FCA-induced arthritis. Complementary behavioural studies were also performed in order to determine the role of $\beta_2$-adrenoceptors in modifying the inflammation and hyperalgesia associated with monoarthritic rats.

*SECTION 2*

# *MATERIALS AND METHODS*

# MATERIALS AND METHODS

## 2.1 Induction of localised adjuvant-induced arthritis

Localised adjuvant arthritis was induced in male Wistar rats (200 - 350g) by the subdermal injection of Freunds Complete Adjuvant (FCA) around the tibio-tarsal joint of the left hind limb. Following induction of anaesthesia using ether or halothane (2% in oxygen), the left hind limb was swabbed with alcohol, and a total volume of 150µl of FCA (1mgml$^{-1}$ heat killed *mycobacterium tuberculosis*, mannide monooleate 0.15ml, paraffin oil 0.85ml) injected subdermally at two separate sites (75µl at each site). Following recovery, the rats were housed in cages (5 rats per cage). The development of inflammation was followed for up to 30 days, during which period the animals were used for electrophysiological or behavioural experiments.

## 2.2 Behavioural studies using arthritic rats

### 2.2.1 Assessment of the arthritic lesion for behavioural studies

Quantitative measurements were obtained of ankle joint circumferences, and foot withdrawal following the application of increasing pressure to the left (adjuvant-injected) or right ankle (un-injected) joint. Qualitative measurements were also made of inflammation and mobility. Rat body weight was used as a general indicator of animal health. The various measurements were performed, blind to drug treatment, by the same experienced investigator and are described in detail below.

1) Ankle joint circumference: this was measured (in mm) by placing a loop of measuring tape around the ankle joints.

2) Foot withdrawal to pressure: rats were hand held, and the ankles of the right and left hind limbs were in turn placed between the thumb of the investigator and a rubber pad. The rubber pad was connected to a spring-loaded linear displacement transducer with the measurement of pressure applied being displayed on a meter. To raise pressure on the ankle joint, the experimenter manually increased the force on the rubber pad. The pressure unit ( 0 - 8; 0 = 1mmHg, 8 = >40mmHg) at which the rat withdrew the hind limb was determined.

3) Mobility (walking foot placement score): rats were observed in the cage to assess any change in gait of the left (adjuvant-injected) and right (un-injected) hind limb. Animals were scored as follows:

0 = normal gait

1 = occasional lifting of foot

2 = foot mostly raised

3 = foot raised all the time (three legged gait)

4) Inflammation: the inflammation present on the left and right ankle joints was determined by visual observation using the following scores:

0 = normal

1 = mild redness

2 = moderate redness

3 = severe redness / lesions

### 2.2.2 Statistical assessment

#### 2.2.2.1 Effects of adjuvant injection

Any difference in the various parameters measured (see Section 2.2.1) between the ipsilateral (adjuvant-injected) and the contralateral (un-injected) ankle joint was assessed statistically using analysis of variance (ANOVA) and the Mann Whitney U-test (see Section 2.2.2.2 for details).

#### 2.2.2.2 Effects of pharmacological agents in adjuvant-arthritic rats

For ankle joint circumferences, withdrawal to pressure or weight measurements, an ANOVA test was first performed. This essentially involved summing the values for each parameter for individual animals. For example, readings such as the left ankle joint circumference for rat 1 in group A were totalled during a defined period to give an integrated value which is equivalent to the "area under the curve". This calculation was repeated for the other rats in the group. In the studies where drug treatment was started before the injection of adjuvant, the defined period was days 2 - 9 and days 9-19 for the acute and secondary phases, respectively. For the studies where rats with an established arthritis were subsequently treated with drugs, the defined period was 14 - 29 days. Adjuvant was injected on day 0 (established arthritis studies) or day 1

(all other studies). The individual summed measurements for drug-treated animals were compared with those for the vehicle-treated group by ANOVA, and the null hypothesis rejected at $P < 0.05$. If the ANOVA was found to significantly show a difference between the groups, then further statistical analysis was performed using the Mann Whitney U-test at varying time points. For the qualitative measurements (inflammation and walking foot placement) the Mann Whitney U-test was used. P values less than 0.05 were considered to be significant.

## 2.3 Electrophysiological experiments *in-vivo*

### 2.3.1 Assessment of the arthritic lesion for electrophysiological studies

The general condition and activity of the rats was monitored on a daily basis by observing their behaviour in relation to motility and stress during handling (see Colpaert, 1987) in the cage. Weight gain was monitored regularly along with visual assessment of any secondary inflammatory lesions in the hind limbs, tail, snout, genitalia and ears. Measurement of ankle joint swelling was made by using a measuring tape around the arthritic joint and compared to the circumference of contralateral (un-injected) ankle joint.

### 2.3.2 Anaesthesia and surgical preparation

Male Wistar rats (weight 312 ± 39g; range: 250-450g) were anaesthetised by intra-peritoneal injection of urethane (25% w/v aqueous ethylcarbamate solution, $0.6ml100g^{-1}$ body weight). Body temperature was monitored continuously and

maintained at 37 ± 0.5°C by an automated electronic heating blanket (Harvard apparatus Ltd) connected to thermistor probe inserted in the rectum.

A midline incision was made in the ventral aspect of the neck, and a tracheal cannula was inserted below the level of the larynx. The left carotid artery was cannulated with a catheter (OD 0.75mm), and arterial blood pressure monitored by connection of the catheter to a pressure transducer (Bell and Howell, 4-442). The signal from the transducer was amplified and displayed on a chart recorder (Devices M4). A cannula (OD 0.63mm) was inserted into the right femoral artery for the injection of drugs at the level of the iliac bifurcation of the abdominal aorta.

The tibial nerve of the left hind limb was exposed through a longitudinal incision in the groin and medial aspect of the leg from the pelvis to the sole of the foot. A free exposure of the nerve was obtained by removing the semitendinosis, semimembranosis, biceps femoris and medial gastrocnemius muscles. The plantar nerves were then exposed by careful incision and reflexion of the crural fascia overlying the stumps of the Achilles tendon and, the primary articulo-cutaneous ramus (PACR) nerve identified where it left the medial plantar nerve (Guilbaud et al., 1985). The dissection required to expose the PACR nerve and to provide sufficient skin flap to form a liquid paraffin pool, adequate enough to cover the exposed nerve during electrical recording, inevitably lead to rupture of the cutaneous branches of the PACR so that neural recording was restricted to activity in nerve fibres innervating the subcutaneous tissue and the ankle joint. Figure 2.1 shows a diagrammatic

representation of the recording arrangement together with a photograph of the working preparation.

### 2.3.3 Recording afferent nerve activity

Electrical activity in afferent nerve fibres was recorded either from fine filaments split from the PACR close to its origin from the medial plantar nerve, or from the intact PACR after it had been dissected away from the adjacent tissues. The tibial nerve was cut centrally to abolish efferent nerve activity in the PACR. Electrical activity was recorded extracellularly using bipolar platinum-iridium wire electrodes mounted on a micromanipulator. The electrodes were carefully placed in contact with the lower side of the nerve filament.

The electrical signal was amplified (Neurolog) and displayed on a storage oscilloscope (Tektronix 5113). The signal was then digitised using a digital audio processor (Sony PCM 701-ES) for storage on the videotape (videotape recorder: Sony Betamax SL-HF100 UB). The output from the amplifier was also passed through a voltage discriminator (Digitimer D130) so that action potentials of a selected amplitude could be counted. Output from the voltage discriminator was also relayed to a loud speaker. Figure 2.2 illustrates the arrangement used for recording and storing data.

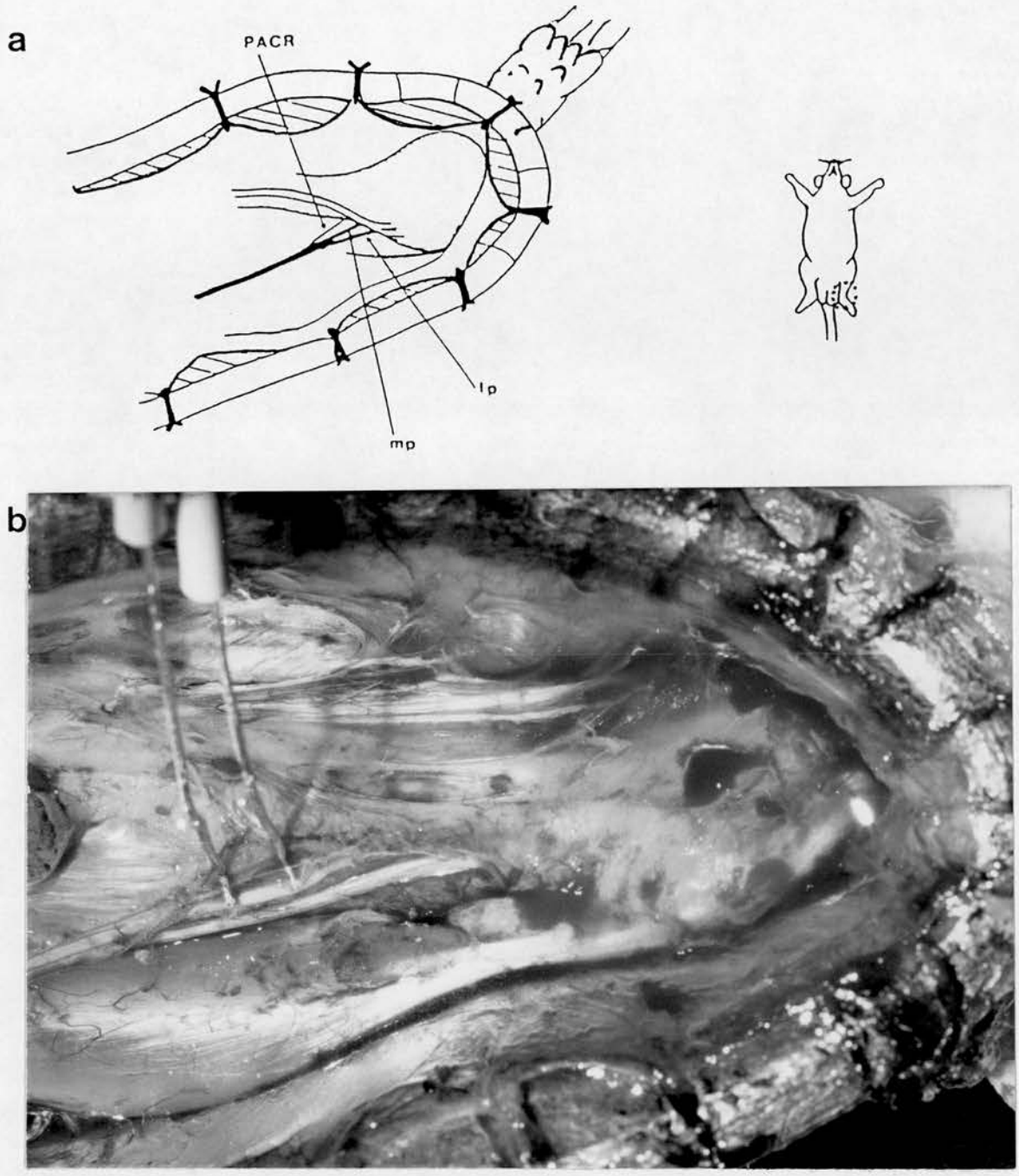



Figure 2.1 (a) Schematic diagram showing the primary articulo-cutaneous ramus (PACR) nerve as prepared for recording purposes. The skin has been dissected from the limb to form a paraffin oil pool, thus exposing the tissues of the ankle joint. Labelled in the diagram is the PACR and the medial (mp) and lateral (lp) plantar nerves. The PACR leaves the medial plantar nerve and passes under the saphenous nerve to innervate the tissues of the joint. (b) Enlarged (x4) photograph of the working preparation. The PACR is hooked up to a pair of platinum-iridium wire electrodes for recording afferent neural activity from the ankle joint.

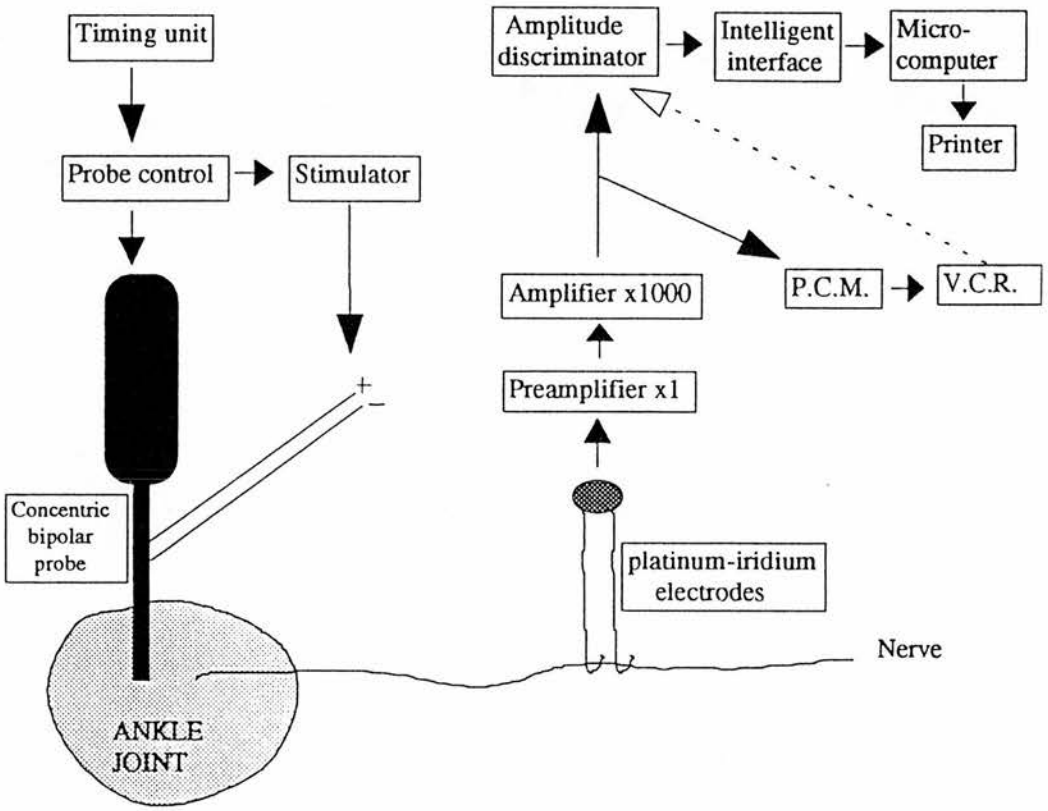



Figure 2.2 Schematic diagram showing the arrangement of apparatus used for electrophysiogical recording, collection and storage of data, and in the application of mechanical stimuli. P.C.M. = pulse code modulation. V.C.R. = video cassette recorder.

### 2.3.4 Mechanical and electrical stimulation

Afferent units were found by exploring the exposed medial aspect of the left ankle joint using a hand held plastic probe of approximately 1mm diameter. Quantitative mechanical stimuli were delivered using an electromechanical indentation generator (Somedic, Sweden). Ramp and plateau stimulus waveforms were routinely used with displacements of between 100-500μm. Indentation stimuli were of two seconds duration, applied at two minute intervals in order to minimise receptor fatigue. Once a receptive field had been found, the threshold force needed to activate the mechanoreceptor was determined by means of a strain gauge attached to the mechanical probe.

The mechanical probe consisted of a silver wire core isolated from the metal cylinder casing of 1mm external diameter. This bipolar arrangement of the probe allowed its use in applying localised electrical stimuli of 1msec pulse duration at 1Hz - voltage (0.1 - 5V) was slowly increased until there was activation of the spike. Conduction velocity (v) was calculated from the conduction distance (d) measured *in situ* and the conduction time (t) for the action potential to travel from the stimulating to the recording electrodes ($v = d / t$, $ms^{-1}$). Overall, this method for conduction velocity measurement is likely to under-estimate the conduction velocity (nerve path longer than measured distance because of 'coiling' in the nerve trunk).

The high level of technical ability required to dissect the PACR nerve for neural recordings resulted in only a proportion of the preparations being functional. On

average a successful neural recording was obtained in approximately 60% of the preparations.

### 2.3.5 Data analysis

The output of the video tape recorder containing the neural recording was passed through an amplifier and filter module (band pass 10 - 1000 Hz) and fed to the spike processor which counted selected action potentials. Each action potential that fell within the window generated a pulse of 1ms duration. The number of impulses occurring in 0.1s intervals was collected and stored by a personal computer (IBM PC) programmed to collect over a period of up to 450s (software written and supplied by Dr M. Dutia, Dept. of Physiology, University of Edinburgh). A marker was used to indicate the point at which a drug injection was made. Collected data were stored on floppy disc and subsequently analysed on the personal computer using the same programme.

A computer-generated plot of the action potential discharge frequency as a function of time was displayed on the computer monitor. From this plot neural discharges were analysed in the pre-injection control period and during defined intervals following injection of the drug. The defined time period (t seconds), for which the computer was required to process discharge frequency was selected using a moving cursor which directed the computer to the memory-stored data displayed on the monitor.

#### 2.3.5.1 Mechanically-evoked stimuli

The responses to mechanical stimuli were expressed as the number of action potentials produced by each 2s indentation stimulus. Drug effects were expressed in terms of the peak number of impulses above or below the pre-injection evoked discharge. In experiments where a large increase in background discharge coincided with the mechanical stimulus it became difficult to quantify the response to the mechanical indentation only. In such cases, the response to the mechanical stimulus was still determined over 2s even although the measurement will over-estimate the actual evoked discharge. Subtraction of the raised background discharge from the response to the mechanical stimulus was investigated but was rejected, since in this calculation negative values could be obtained for the response to the mechanical stimulus.

#### 2.3.5.2 Spontaneous discharge

When analysing the actions of drugs on on-going or spontaneous discharge, the control period was defined as the 60s period immediately prior to the drug injection. Drug effects were quantified either as the mean peak discharge (measured over 10-15s with the exception of indomethacin, where successive 100s time periods were used) above or below the mean pre-injection discharge (see analysis A below), or, as with drugs showing small responses, the total number of action potentials above or below the control rate of discharge (see analysis B below). The calculations used for such quantifications were as follows.

**Preliminary definitions:**

i) $\Sigma x$: the total number of impulses (number of action potentials) occurring in the period t seconds, for control and for test periods.

Units: impulses.

ii) $\bar{x}$: the mean number of impulses per second occurring in each period t seconds

Hence $\bar{x}$ (control) = $\Sigma x$ (control) / t (control),

and $\bar{x}$ (test) = $\Sigma x$ (test) / t (test).

Units: impulses per second (i.p.s.).

**Analysis A:**

**delta x**: the difference in the mean discharge occurring between the test and control periods. Thus,

delta $\bar{x}$ = $\bar{x}$ (test) - $\bar{x}$ (control).

Units: i.p.s.

**Analysis B:**

**delta $\Sigma x$**: the absolute difference in discharge from control levels, given by

delta $\Sigma x$ = $\Sigma x$ (test) - [$\bar{x}$ (control) $*$ t (test)],

Units: impulses

with the assumption that the pre-injection discharge remains constant in the absence of any modifying effect of the drug. Such an assumption is supported by the finding that control injections of saline produced no significant change in discharge ($P>0.05$, Wilcoxon, versus pre-injection discharge).

Whereas analysis A takes only the magnitude of the response into account, data integrated with respect to control (delta $\Sigma x$, analysis B) takes into account both the magnitude and duration of the induced change in discharge. The results of analysis A and B will have positive and negative values when there is enhancement and depression of neural discharge, respectively.

### 2.3.6 Statistical assessment

Comparison of neural discharges obtained in normal and arthritic rats was assessed using the Mann Whitney U-test. The Wilcoxon signed rank (Wilcoxon) was used to determine the statistical significance of drugs at producing changes in neural discharge as compared to the pre-injection control discharge period. P values less than 0.05 were considered to be statistically significant.

### 2.3.7 Drug administration

All drugs were injected intra-arterially (i.a.) in a volume of 0.1 - 0.3ml into the bifurcation of the abdominal aorta via the cannula inserted retrogradely through the right femoral artery, and washed in with 0.2ml saline (0.9% w/v aqueous NaCl). Bolus injections made in this way resulted in a transient high local concentration of the drugs at the left ankle joint. Injections were completed within a period of 1s. All drugs were administered 15s prior to a mechanical stimulus so that any short-lasting actions could be observed. If a drug produced a change in neural discharge, then the next dose of the drug was only injected after neural discharge had returned to or near the control resting discharge frequency; an exception to this was the studies in which indomethacin was used (see Section 4). Control injections of 0.1ml saline, washed in with 0.2ml saline, were made in each experiment. In all cases, injection of saline had no significant effect on afferent discharge (spontaneous or mechanically-evoked). Prior to the administration of drugs, consistent responses to the application (every 2min) of the mechanical stimulus were obtained when possible. Log dose-response curves for agonists were constructed (when possible) before and after antagonists or enzyme inhibitors.

## 2.4 Isolated nerve preparation

### 2.4.1 Preparation of tissues

Adult male Wistar rats (200-350g) were killed by cervical dislocation. Segments of vagus, tibial, tibialis, sciatic or saphenous nerve of approximately 10 - 15mm were

dissected out and placed in Krebs solution of the following composition (mM): NaCl 118, KCl 5.4, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_2$ 25, glucose 11.1 and $CaCl_2$ 2.5. The Krebs solution was gassed with 95% $O_2$ and 5% $CO_2$ and maintained at 27°C. The connective tissue sheath around each nerve segment was carefully removed using watchmaker forceps.

### 2.4.2 Extracellular recording

Within one hour of dissection, sections of desheathed nerve were transferred to two compartment perspex baths to permit extracellular recording of membrane potential changes. Each nerve was positioned so that approximately half of its length lay in the first compartment, while the remainder projected through a greased (Dow-Corning high vacuum grease) slot into the second. The DC potential between the two compartments was recorded via silver-silver chloride electrodes connected to the tissue preparation via agar-saline / filter paper bridges and was displayed on a potentiometric chart recorder (Rikadenki). The perfusate containing Krebs with or without the test drugs was dripped directly onto the tissues at a rate of 1 - 1.5ml$min^{-1}$. Drugs were applied at a known concentration via the superfusion stream into the first compartment with a tissue contact time of 3 - 5min followed by drug washout using drug-free Krebs for 15min. A diagrammatic representation of the preparation is shown in Figure 2.3.

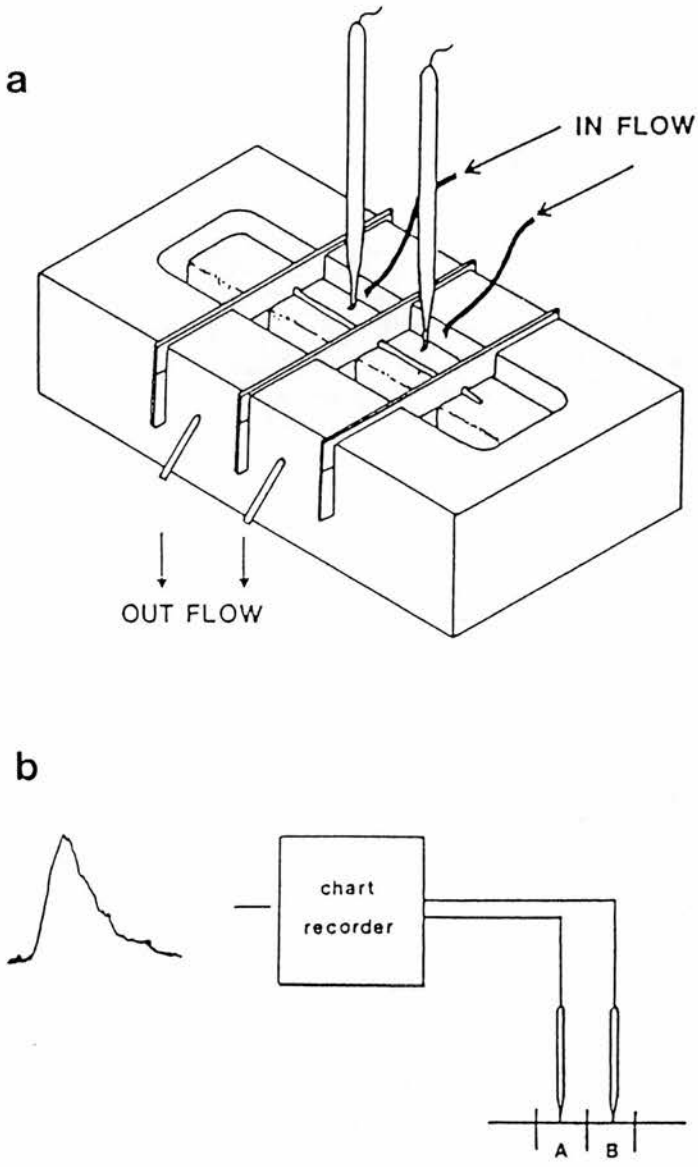



Figure 2.3 (a) Apparatus for recording extracellular changes in membrane potential from isolated nerves. (b) Layout of recording apparatus. The continuous chart record displays the whole nerve membrane potential when recording differentially from chambers A and B.

### 2.4.3 Experimental protocol

An attempt was made to obtain Log concentration-response curves to bradykinin and des-Arg$^9$-bradykinin (0.01- 10000μM). To test the hypothesis that bradykinin receptors could be induced in the isolated nerves, a protocol was devised where a single concentration of bradykinin or des-Arg$^9$-bradykinin (0.1μM) was applied repeatedly over 4 - 5hr; contact time was for 3 - 5min followed by drug washout using drug-free Krebs for 20min. The effects of superfusing a combination of bradykinin (0.1μM) and $PGE_2$ (1μM) was also determined. To confirm that the preparations were indeed capable of showing changes in membrane potential a depolarising concentration of KCl (10mM - 100mM) was used either before, but more typically, after perfusion of the test drugs.

## 2.5 Rat vas deferens preparation

### 2.5.1 Preparation of isolated vas deferens

Adult male Wistar rats (200-350g) were killed by cervical dislocation and the vasa deferentia immediately removed. The surrounding connective and adipose tissue and the adjoining blood vessels were removed from the vas deferens. Only the prostatic segment (1-1.5cm) of the vas deferens was used in the experiments.

Each prostatic segment was immersed in a 5ml organ bath containing Krebs solution of the following composition (mM): NaCl 118, KCL 5.4, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2,

$NaHCO_2$ 25, glucose 11.1 and $CaCl_2$ 2.5. The Krebs solution was gassed with 95% $O_2$ and 5% $CO_2$ and maintained at 35°C.

Each prostatic segment was suspended between two parallel platinum electrodes which were used for transmural electrical stimulation. Electrical pulses were delivered by a multi-stimulator (Digitimer System-D330) at a frequency of 0.33Hz, 1msec pulse duration and at supramaximal voltage. To record isometric muscular contractions, the segments were connected to a force transducer (Dynamometer UF1) coupled to a chart recorder (Lectromed MT8-PX).

Prior to electrical field-stimulation or the application of drugs, the tissues were allowed to equilibrate for 60min with 0.5g of basal tension. During this equilibration period, the tissues were washed with Krebs solution every 15min and the tension re-adjusted to 0.5g.

### 2.5.2 Agonist / antagonist protocols

Agonists were added to the field-stimulated vas deferens in a cumulative manner. After completion of a cumulative-concentration response curve, field-stimulation of the tissues was stopped and the vas deferens washed with Krebs solution every 2min for 10min. A further 20min equilibration period (no electrical stimulation) was allowed before constructing the next cumulative concentration-response curve.

To evaluate the potency of the bradykinin (BK) antagonists, cumulative concentration-response curves to BK were constructed in the absence and presence of the test antagonist. Antagonists were added to the organ baths, in the absence of electrical stimulation, 20min prior to the construction of the BK concentration-response curves. Three successively increasing concentrations of antagonist were equilibrated with the vas deferens, with each concentration of antagonist being washed out before the addition of the next higher concentration. In another series of BK antagonist studies, BK cumulative concentration-response curves were constructed before and after co-incubation of NPC349 (30 and 100μM) at varying time periods with Hoe140 (30nM).

Antagonist washout experiments were performed by constructing cumulative concentration-response curves to BK before and after the addition of a high concentration of the BK antagonist. To investigate the specificity of the BK antagonists in the vas deferens, neurogenic and musculotropic response curves were constructed to noradrenaline, angiotensin II and U46619 before and after the addition of the BK antagonists.

In preliminary studies, neurogenic and musculotropic cumulative concentration-response curves to BK, were constructed before and after prazosin, propranolol, atropine, mepyramine, ranitidine, thioperamide, ondansetron, GR113808, GR82334, Men10207, indomethacin and staurosporine (0.3-1μM). These various agents were also added (1μM) during basal electrically-evoked stimulation of the rat vas deferens.

In another series of preliminary experiments, the $P_2$ receptor was desensitised by two successive additions of α,β-methylene-ATP (10μM). The effects of basal electrically-evoked stimulation, BK (1μM), ATP (1mM) and U46619 (1μM) were obtained before and after inducing tachyphylaxis to α,β-methylene-ATP.

### 2.5.3 Data analysis

Increases in basal muscle tension (musculotropic response) are expressed as a percentage of the maximal response that was attained by the test agent. The potentiations in the magnitude of the electrically-driven twitches (neurogenic response) are quantified as the percentage of the maximal response above the basal twitch attained by the test agent. The potency of BK and the $B_2$ receptor agonists at producing the neurogenic and musculotropic responses are expressed as the geometric mean (95% confidence limits) of the $EC_{50}$ (concentration which produces 50% of the maximal response) values.

In the BK antagonist studies, $pA_2$ and slope values were obtained from Schild plots using a curve fitting program (Baspak, Glaxo Research & Development). Incubation with Hoe140 decreased the maximal BK musculotropic response (insurmountable antagonism) such that conventional Schild analysis could not be used to determine the potency of the antagonist. Nevertheless, an apparent $pK_B$ for Hoe140 was derived using a double regression plot (Kenakin, 1984). Essentially, such a plot involves plotting 1/A vs 1/A' where A and A' are the equieffective concentrations of agonist in

the absence and presence of Hoe140, respectively. An estimated $pK_B$ is then derived by using the gradient (G) of this plot in the Gaddum Equation ($pK_B$ = -log ([B] / G-1), where B is the antagonist concentration).

### 2.5.4 Statistical analysis

Experimental values are given as the mean ± s.e.mean. *n* represents the number of vas deferens used. For the BK agonist studies, the student's paired t-test was used to determine statistical differences between the neurogenic and musculotropic $EC_{50}$ values of each test agent. The un-paired t-test was used to determine statistical relevance between BK and $B_2$ receptor agonists at the neurogenic and at the musculotropic responses. For the $B_2$ receptor antagonist studies, the paired t-test was used to statistically compare the $pA_2$ and slope values at the neurogenic response with those obtained respectively at the musculotropic response. P values less than 0.05 were considered to be statistically significant.

*SECTION 3*

# *ASSESSMENT OF ADJUVANT-INDUCED MONOARTHRITIS IN THE RAT ANKLE JOINT.*

## 3.1 INTRODUCTION

Adjuvant-induced polyarthritis is a widely used and accepted model of arthritis in the rat, which is associated with chronic pain and inflammation (see Billingham, 1983; Colpaert, 1987). This form of arthritis is induced by injecting heat-killed mycobacteria (suspended in mineral oil) into the tailbase or footpad with the resulting arthritis being a whole-animal disease affecting multiple joints with lesions in the eyes, ears, nose, skin, tail and genitals as well as profound weight loss (Pearson & Wood, 1959; Ward & Jones, 1962; Pearson & Wood, 1963; Rainsford, 1982). The severest inflammation in polyarthritis occurs in the joints of the hind limbs where there is bone destruction, damage to tendons and loss of cartilage.

Although the use of adjuvant-induced polyarthritis is justifiable in many neurophysiological, biochemical and immunological studies, in the study of nociceptive mechanisms the systemic and widespread nature of the arthritis makes it difficult to attribute observed changes specifically to the arthritis process. There are also ethical considerations in the use of such severely affected animals. Therefore, it is desirable both on scientific and ethical grounds to use a less severe and more localised adjuvant-induced arthritis as an alternative to adjuvant-induced polyarthritis in the study of nociceptive processes. Recent studies have shown that a discrete monoarthritis is obtained in rats following the localised injection of Freunds adjuvant around (subdermally) the ankle joint (Grubb et al., 1991; Donaldson et al., 1993).

The aims of the present experiments were to verify the induction of monoarthritis in the rat ankle joint by injecting locally adjuvant around (subdermally) the ankle joint, and then to further assess this unilateral model of arthritis in behavioural, histopathological and electrophysiological studies.

## 3.2 MATERIALS & METHODS

### 3.2.1 Adjuvant-induced monoarthritis

Following induction of anaesthesia in male Wistar rats (200 - 250g) using ether or halothane (2% in oxygen), a total volume of 150µl of Freunds complete adjuvant (FCA, 1mgml$^{-1}$ heat killed *mycobacterium tuberculosis*, mannide monooleate 0.15ml, paraffin oil 0.85ml) was injected subdermally at two separate sites (75µl at each site) around the left ankle joint, and the rats allowed to recover from the anaesthesia. Rats were housed five to a cage. The development of inflammation was followed for up to 30 days.

Quantitative measurements were obtained of ankle joint circumferences, and foot withdrawal following the application of pressure to the left (adjuvant-injected) and right (un-injected) ankle joints. Qualitative measurements were made of joint inflammation and mobility. Details of these various quantitative and qualitative measurements are described in Section 2.2.1.

### 3.2.2 Electrophysiological studies

The in-vivo preparation, neural recording and off-line analysis are described in detail in Section 2.3. In brief, male Wistar rats (normal and adjuvant-arthritic) were anaesthetised with urethane and cannulations performed of the trachea, right carotid artery (blood pressure monitoring) and right femoral artery (retrograde cannulation for close intra-arterial bolus injection of drugs into left limb). The medial aspect of the

left ankle joint was exposed and nerve fibres were isolated from the PACR nerve. C-fibre afferent discharge (spontaneous and mechanically-evoked) from articular mechanonociceptors was recorded extracellularly, using bipolar platinum-iridium electrodes.

Afferent mechanosensitive units were identified by exploring the exposed medial aspect of the left ankle joint using a hand held perpex probe of approximately 1mm diameter. Quantitative mechanical stimuli were delivered using an electromechanical indentation generator. Ramp and plateau stimulus waveforms were routinely used with displacements of between 100-500μm. Indentation stimuli were of two seconds duration, applied at two minute intervals in order to minimize receptor fatigue. Once a receptive field had been found, the threshold force needed to activate the mechanoreceptor was determined by a strain gauge attached to the mechanical probe. The mechanical probe consisted of a silver wire core isolated from the metal cylinder casing of 1mm external diameter. This bipolar arrangement of the probe allowed its use in applying localised electrical stimuli. Conduction velocity (v) was calculated from the conduction distance (d) measured *in situ* and the conduction time (t) for the action potential to travel from the stimulating to the recording electrodes ($v = d / t$, $ms^{-1}$).

### 3.2.3 Effects of capsaicin on afferent neural discharge

In all the *in-vivo* electrophysiological recordings from C-fibre afferent mechanonociceptors in both normal and arthritic joints, chemosensitivity of units was

tested by a low dose of the C-fibre excitant, capsaicin (1μg, i.a.), injected at the start and /or end of each experiment. The effects of capsaicin (1μg, i.a.) on mechanically-evoked discharge was also determined. In preliminary studies, the increase in afferent neural discharge induced by capsaicin (3μg, i.a.) was investigated before and after injection of the novel capsaicin receptor antagonist, capsazepine ($1 mgkg^{-1}$, i.a.). As capsazepine has a very short duration of action *in-vivo* (Dr. M.N. Perkins, personal communication) it was injected 2 - 5s prior to the injection of capsaicin (3μg, i.a.).

**Data analysis**

In this series of experiments, capsaicin-induced increase in spontaneous discharge was assessed by determining the mean peak discharge (determined over 10s) above the pre-injection control discharge level (see Section 2.3.5.2 for further details of the formula used) with the delay to onset and the duration of the response also being determined. The control period was defined as the 60s period immediately prior to the addition of capsaicin. A significant increase in spontaneous discharge was defined as an increase over basal discharge of greater than 1 i.p.s. for both normal and arthritic joints. This value of 1 i.p.s. was derived from the results of saline injections in normal ($0.6 \pm 0.1$ i.p.s above basal, 115 units) and arthritic ($0.9 \pm 0.1$ i.p.s. above basal, 198 units) joints. Effects of capsaicin on the responsiveness of the standard mechanical stimulus were assessed as the peak number of impulses above the pre-injection evoked discharge. A significant increase in mechanically-evoked discharge was defined as an enhancement of more than 5 impulses above the pre-injection evoked discharge. This value of 5 impulses was derived from the results of saline injections in

normal (2 ± 1 impulses above basal evoked discharge, 65 units) and arthritic (3 ± 2 impulses above basal evoked discharge, 123 units) joints.

## 3.3 RESULTS

### 3.3.1 Gross pathology and behaviour of monoarthritic rats

The subdermal injection of Freunds complete adjuvant (FCA, 150μg) around the left ankle joint produced a swelling within a few hours. This ankle joint swelling continued to increase for 1 - 3 days (phase I) post-adjuvant before beginning to subside 4 - 8 days (phase II) post-adjuvant (Figure 3.1). From 8 days post-adjuvant (chronic phase) swelling increased over 14 days (phase III), after which a plateau (phase IV) was reached and maintained (Figure 3.1). These phases were also observed for all the other measurents / assessments (see Figure 3.1 - 3.4). Ankle joint circumferences of adjuvant-injected joints were significantly greater than the corresponding ankle joint circumferences of contralateral un-injected joints (Figure 3.1). Inflammation scores of adjuvant-injected left ankle joints were significantly higher as compared with scores from the contralateral uninjected right ankle joints (Figure 3.2). Pressure thresholds for hind limb withdrawal were significantly lowered in the adjuvant-injected left limb as compared to the contralateral uninjected right limb (Figure 3.3). A small reduction, from approximately 11 days and onwards, was observed for the right ankle joint pressure scores, although these scores were still significantly different from those measured in the left ankle joint (Figure 3.3). Walking (mobility) left foot placement scores in rats with adjuvant-injected left ankle joints were significantly higher than the corresponding scores from the contralateral un-injected right hind limb (Figure 3.4).

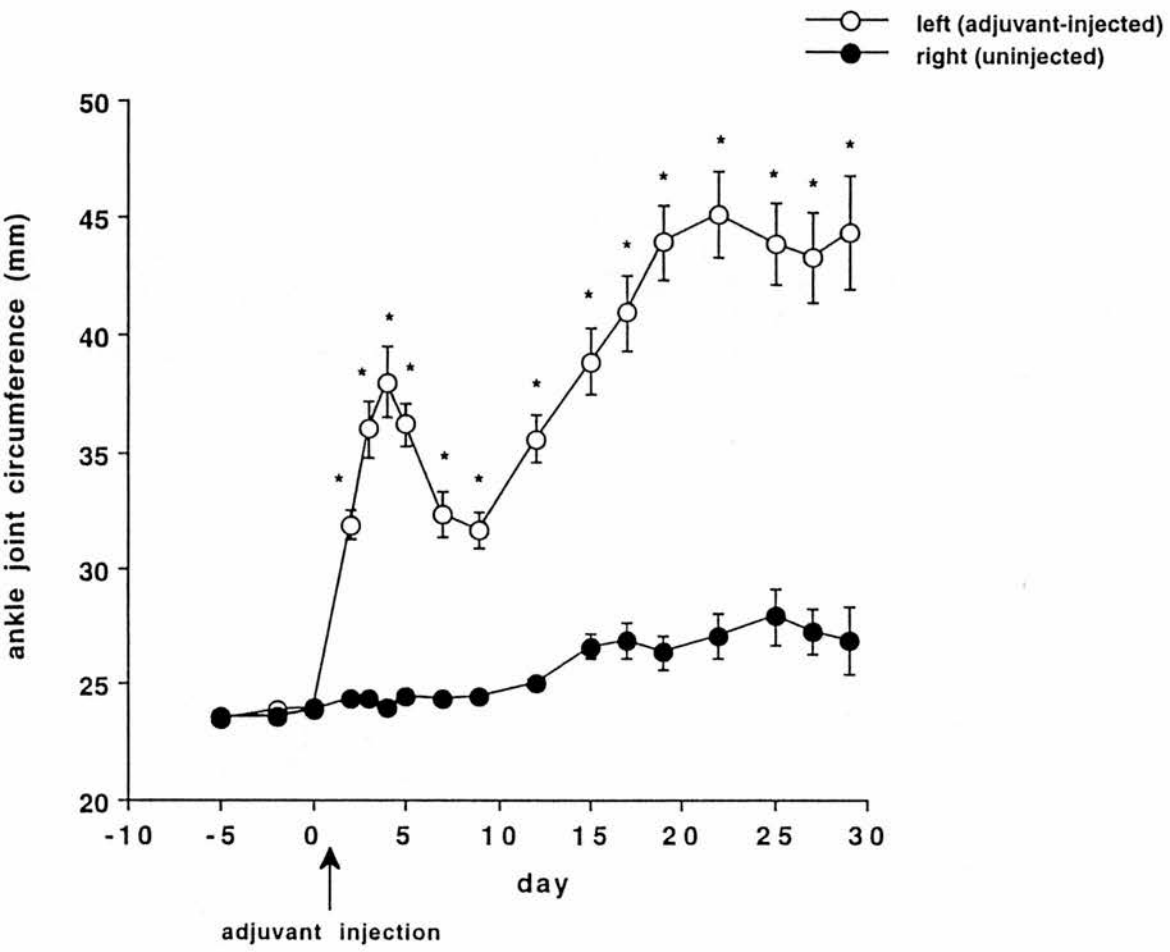



Figure 3.1 Effects of injecting FCA (150μg) around the left ankle joint on ankle joint circumferences. Each point is the mean ± s.e.mean from n=16-24. Statistical analysis: $P<0.05$ ANOVA days 2-9 and 9-29. * $P<0.05$ Mann Whitney U-test, versus right (uninjected) ankle joints.

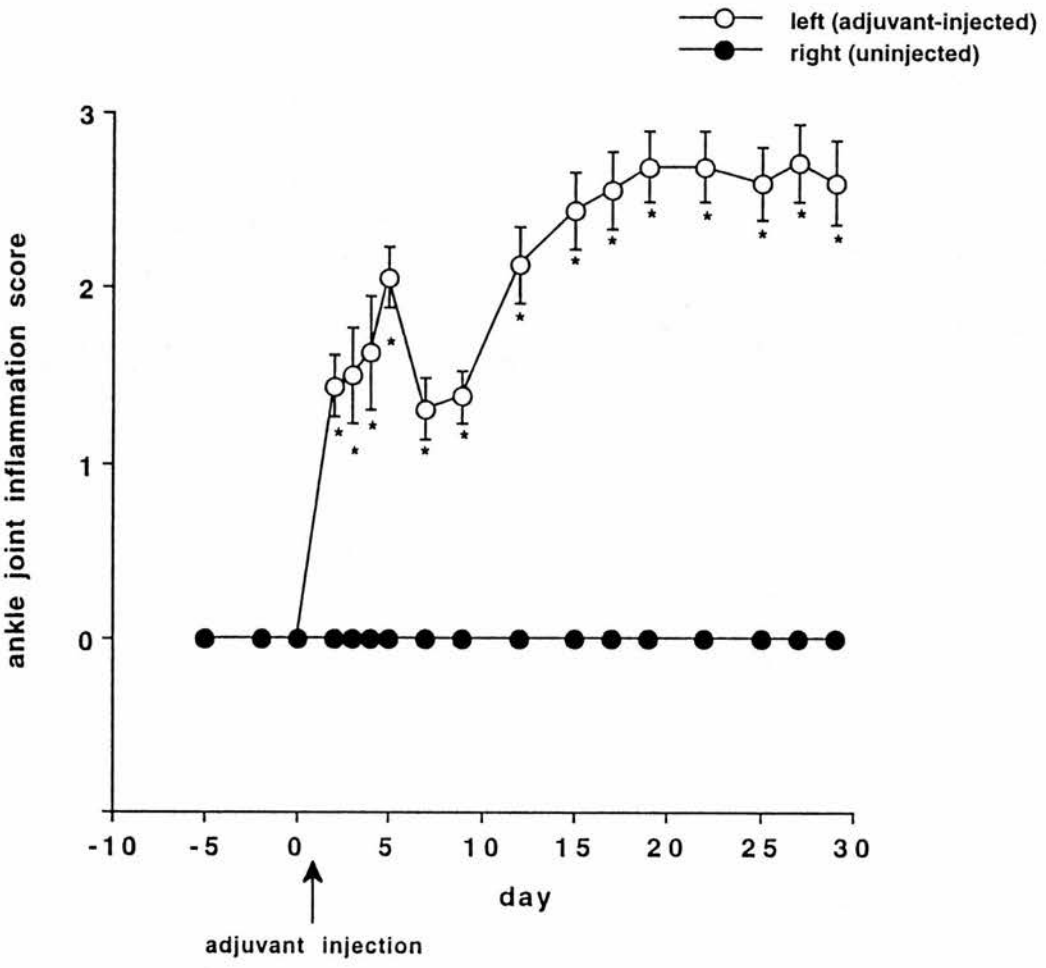



Figure 3.2 Effects of injecting FCA (150μg) around the left ankle joint on ankle joint inflammation scores. Each point is the mean ± s.e.mean from n=16-24. Statistical analysis: * P<0.05 Mann Whitney U-test, versus right (uninjected) ankle joints.

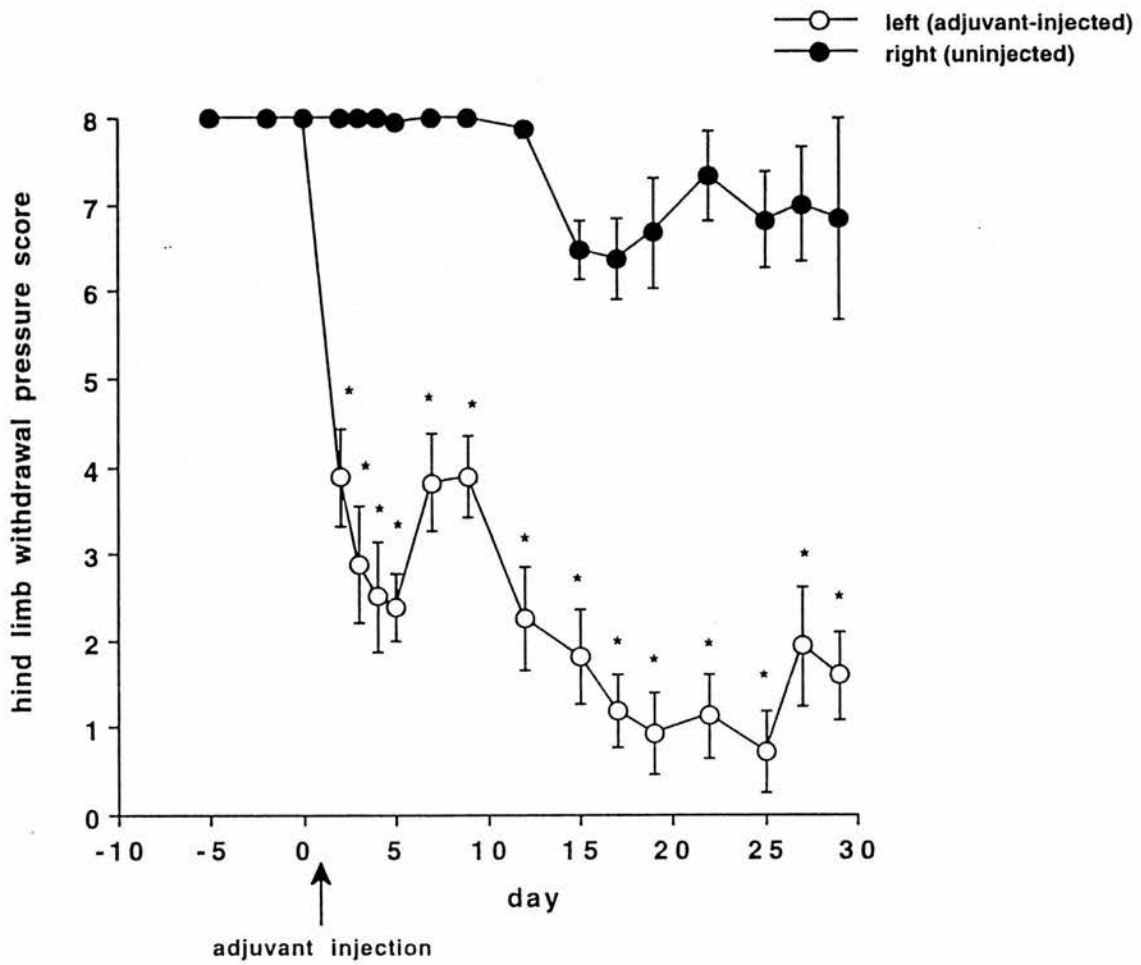



Figure 3.3 Effects of injecting FCA (150μg) around the left ankle joint on hind limb withdrawal pressure scores. Each point is the mean ± s.e.mean from n=16-24. Statistical analysis: $P<0.05$ ANOVA days 2-9 and 9-29. * $P<0.05$ Mann Whitney U-test, versus right (uninjected) ankle joints.

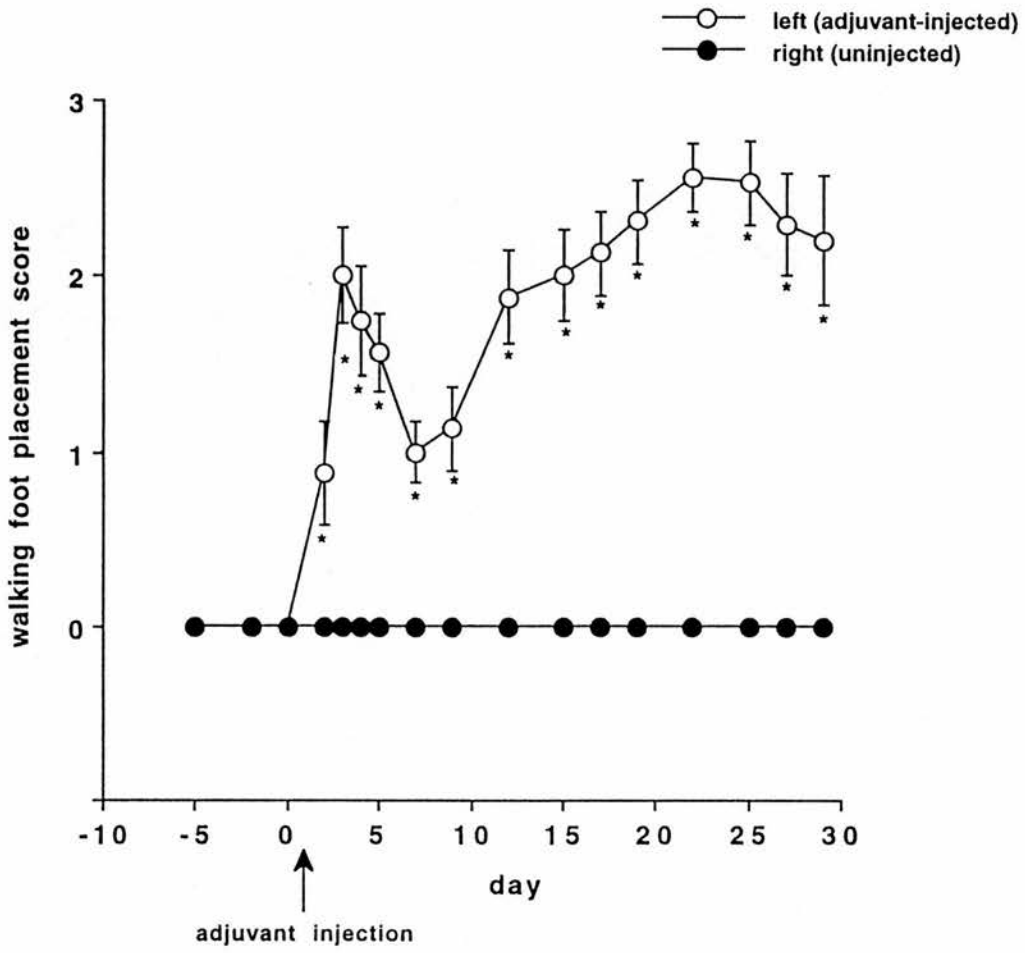



Figure 3.4 Effects of injecting FCA (150μg) around the left ankle joint on walking foot placement scores. Each point is the mean ± s.e.mean from n=16-24. Statistical analysis: * P<0.05 Mann Whitney U-test, versus right (uninjected) ankle joints.

### 3.3.2 Histopathology of adjuvant-induced monoarthritis

Dissection of the left (adjuvant-injected) hind limb revealed the presence of fibrous tissue, which bound together the inflamed muscles, tendons, fascia and skin. The capsular tissues around the tibio-tarsal joint were markedly enlarged and thickened. In contrast, none of these visible features was observed in the uninjected contralateral hind limb.

### 3.3.3 *In-vivo* electrophysiology of normal and adjuvant-arthritic joints

Mechanosensitive receptive fields of the rat ankle joint were identified by mechanical probing with a blunt hand-held perspex probe. In both normal and arthritic joints, individual mechanosensitive units were represented by only a single receptive field, each being approximately 1 - 2mm in diameter. In order to give a standard mechanical stimulus (every 2min) a mechanical indentation generator was used; the mechanical stimuli were of ramp and plateau waveform. Repetition (2min apart) of mechanical stimuli led to a slow steady decline in the unit response with time. The responses of mechanosensitive units in arthritic joints to repeatedly applying mechanical stimuli were generally more susceptible to fatigue than units recorded from normal joints. From 30 normal joints, 68 slowly adapting mechanosensitive units, with receptive fields in the ankle joint, were identified. In contrast, an approximately three-fold greater number (223 units from 30 joints) of slowly adapting mechanosensitive units were identified in arthritic joints. The mechanical activation thresholds of mechanoreceptors in normal joints were generally high, whereas in contrast, thresholds in arthritic joints were considerably lower (Figure 3.5). Mechanoreceptors

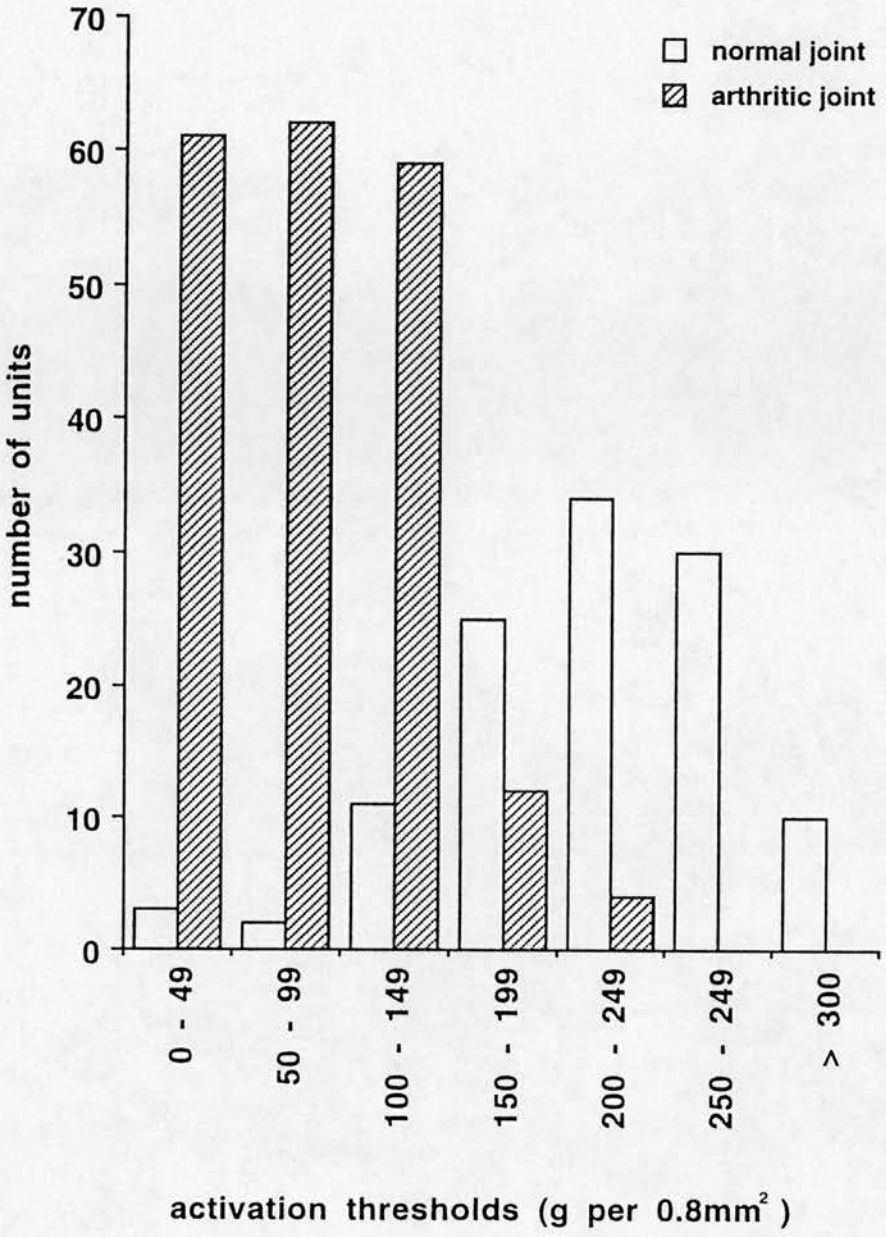



Figure 3.5 Mechanical activation thresholds for mechanonociceptors in normal (115 units from 89 experiments) and arthritic (198 units from 128 experiments) ankle joints obtained using a stainless steel mechanical probe (tip diameter 1mm) with attached strain gauge (see Section 2.3.4).

in arthritic joints were easily found and could be activated by the application of light mechanical pressure, whereas in normal joints the higher mechanical activation thresholds often made finding receptive fields difficult, with compression of the articular tissues against the underlying bone often being the only effective stimulus.

The mean afferent conduction velocity was in the C-fibre afferent range for all units examined both in normal ($0.70 \pm 0.49$ $ms^{-1}$; range 0.22 - 1.50 $ms^{-1}$, 58units) and arthritic ($0.76 \pm 0.54$ $ms^{-1}$; range 0.20 - 1.35 $ms^{-1}$, 76units) joints. No significant difference was found between the afferent conduction velocities in normal and arthritic joints ($P>0.05$, Mann Whitney U-test).

Single afferent units studied in normal joints had low levels of resting (spontaneous) discharge from mechanonociceptors ($0.96 \pm 0.09$ i.p.s.; range: 0 - 1.8, 115units). In contrast, spontaneous discharge was significantly greater (approximately 3 fold, $3.40 \pm 0.46$ i.p.s.; range: 1.4 - 11.6, 198units) in single unit afferents recorded from arthritic joints ($P<0.05$, Mann-Whitney U-test in comparison with normal joints).

Afferent units in both normal and arthritic joints generally had action potential amplitudes of <40μV and spike widths of 2- 3ms.

### 3.3.4 Effects of capsaicin on mechanonociceptor discharge in normal and arthritic joints

In order to test the chemosensitivity of C- fibre afferent mechanonociceptors, a close arterial injection of a low dose of the selective C-fibre excitant, capsaicin (1μg), was

made. In the vast majority (> 95%) of units recorded in both normal and arthritic joints, capsaicin caused an increase (excitation) in spontaneous discharge (see typical response in Figure 3.6 & pooled data in Table 3.1). The small percentage (<5%) of units which were apparently capsaicin (1 - 30μg, i.a.)-insensitive but responsive to mechanical stimuli were not examined further in the present studies. Capsaicin-induced excitation from mechanonociceptors in arthritic joints was significantly greater and of longer duration than that obtained from mechanonociceptors in normal joints (Table 3.1). Capsaicin (1μg, i.a.) also caused an increase in the responsiveness (sensitisation) of mechanonociceptors to the application of the standard mechanical stimulus in approximately 60% and 70% of units examined in normal and arthritic joints, respectively (typical response in Figure 3.6 and pooled data in Figure 3.7); there was either a slight decrease (< 5 impulses) or no change in the mechanical responsiveness in the remaining percentage of units studied. The capsaicin-evoked mechanical sensitisation in arthritic joints was significantly larger (Figure 3.7) and of longer duration (Figure 3.8) than that observed in joints from normal rats.

### 3.3.5 Effects of capsazepine

In preliminary studies, both the capsaicin (3μg, i.a.)-induced excitation, and the sensitisation of mechanically-evoked responses recorded in two units (two experiments) from arthritic joints, were antagonised by the capsaicin receptor antagonist, capsazepine ($1 mg kg^{-1}$, i.a.) (Figures 3.9 - 3.10). Since capsazepine was injected 2 - 5s prior to the injection of capsaicin (because it has a short duration of action *in-vivo,* Dr. M.N. Perkins, personal communication) it was not possible to

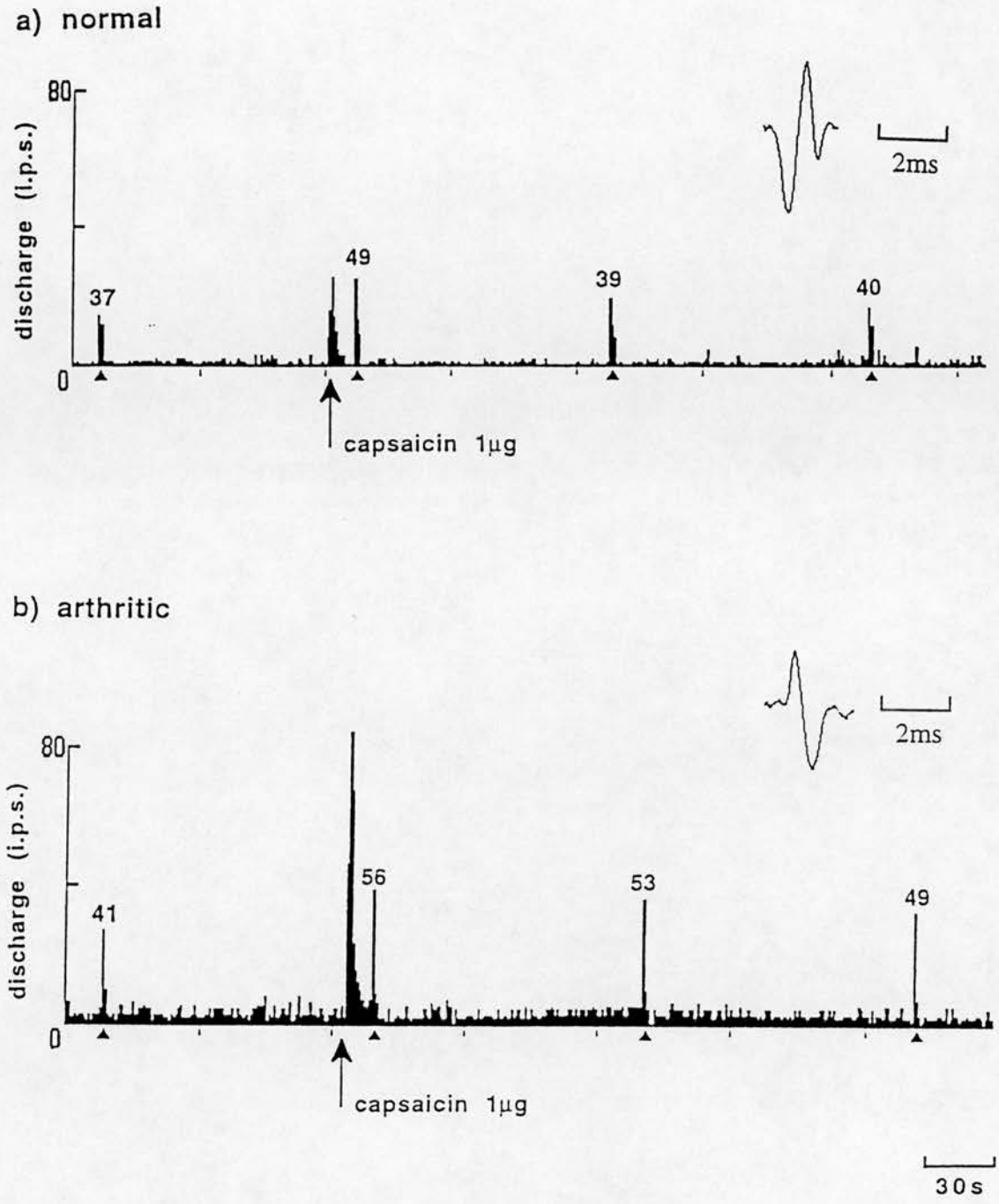



Figure 3.6 Computer generated plot showing capsaicin (1µg, i.a.)-induced excitation and sensitisation of a single C-fibre unit in the (a) normal (conduction velocity $0.5ms^{-1}$) and (b) arthritic (conduction velocity $0.7ms^{-1}$, 16 days post-adjuvant) rat ankle joint. Each bar represents a 1s time interval. Arrowheads indicate the application of the mechanical stimulus. The number of impulses evoked by the mechanical stimulus is given above each response. The insets show fast oscilloscope sweeps of the unit counted.

**Table 3.1 Effects of capsaicin (1μg, i.a.) on spontaneous discharge recorded from articular mechanonociceptors in normal and arthritic rat ankle joints.**

| | *units (experiments)* | *mean peak increase above basal dicharge (i.p.s.)* † | *delay to onset (s)* | *duration (s)* |
|---|---|---|---|---|
| **normal joints** | 115 (89) | 6.88 ± 0.95 | 1.58 ± 0.17 | 11.27 ± 0.62 |
| **arthritic joints** | 198 (128) | 12.55 ± 1.93 * | 1.51± 0.13 | 16.12 ± 1.69 * |

* P<0.05 Mann Whitney U-test, versus value for normal joint. † mean discharge was determined over a 10s time period. The pre-injection basal spontaneous discharge was 0.73 ± 0.11 for normal joints and 3.40 ± 0.46 for arthritic joints.

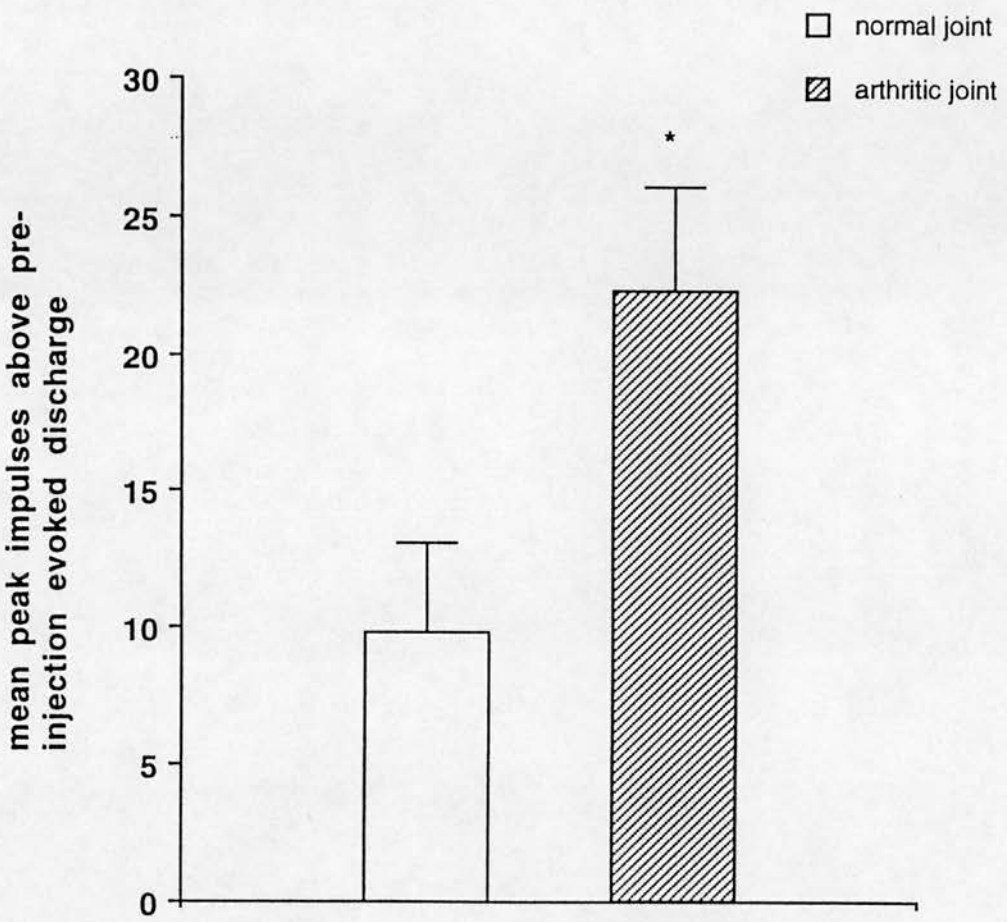



Figure 3.7 Capsaicin (1μg, i.a.)-induced enhancement in the responsiveness to the mechanical stimulus of mechanonociceptors in normal (65units from 40 experiments) and arthritic (123units from 80 experiments) ankle joints. The pre-injection mechanically-evoked discharge was 44 ± 5 and 39 ± 4 impulses for units recorded in normal and arthritic joints, respectively. * $P<0.05$, Mann Whitney U-test, versus normal joint.

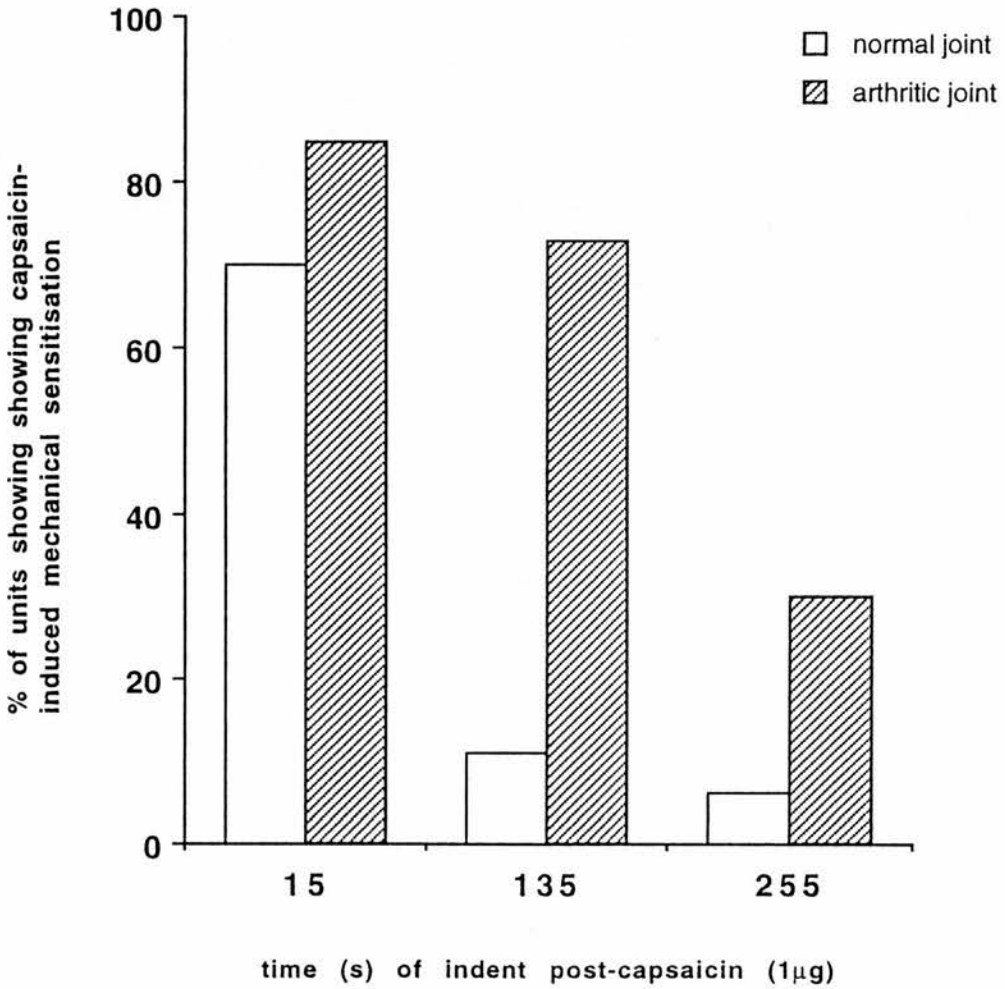



Figure 3.8 Duration of capsaicin (1μg, i.a.)-induced sensitisation of mechano-nociceptors to mechanical stimuli in normal and arthritic joints.

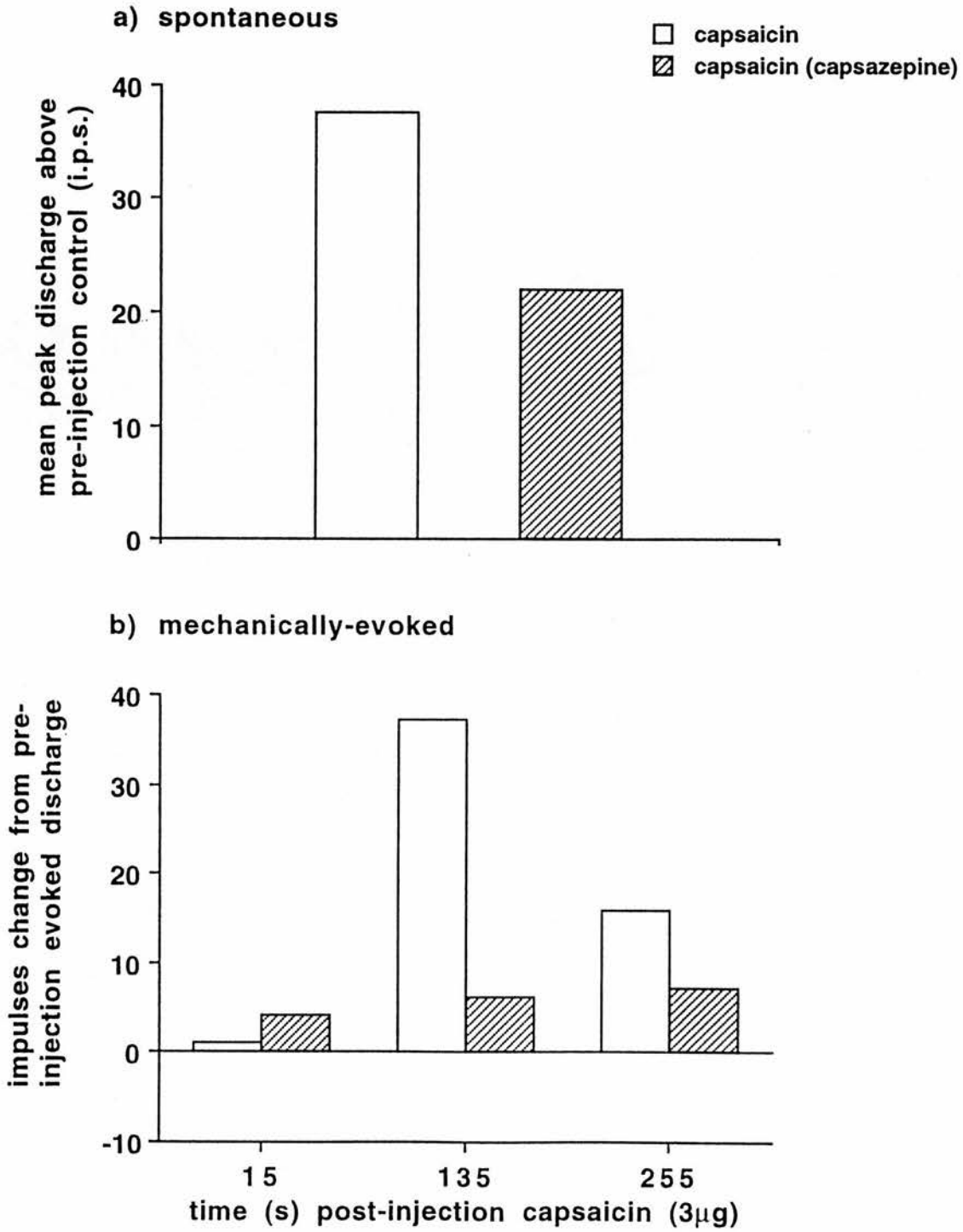



Figure 3.9 Effects of capsaicin (3μg, i.a.), before and after capsazepine (1mgkg$^{-1}$, i.a.), on (a) spontaneous and (b) mechanically-evoked discharge from a single C-afferent fibre unit (conduction velocity 0.33ms$^{-1}$) in an arthritic (40 days post-adjuvant ) ankle joint. The pre-injection spontaneous discharge, was 4.2 i.p.s pre-capsaicin and 5.1 i.p.s pre-capsaicin in the presence of capsazepine. The pre-injection mechanically-evoked discharge, was 104 impulses pre-capsaicin and 90 i.p.s pre-capsaicin in the presence of capsazepine. See also Figure 3.10

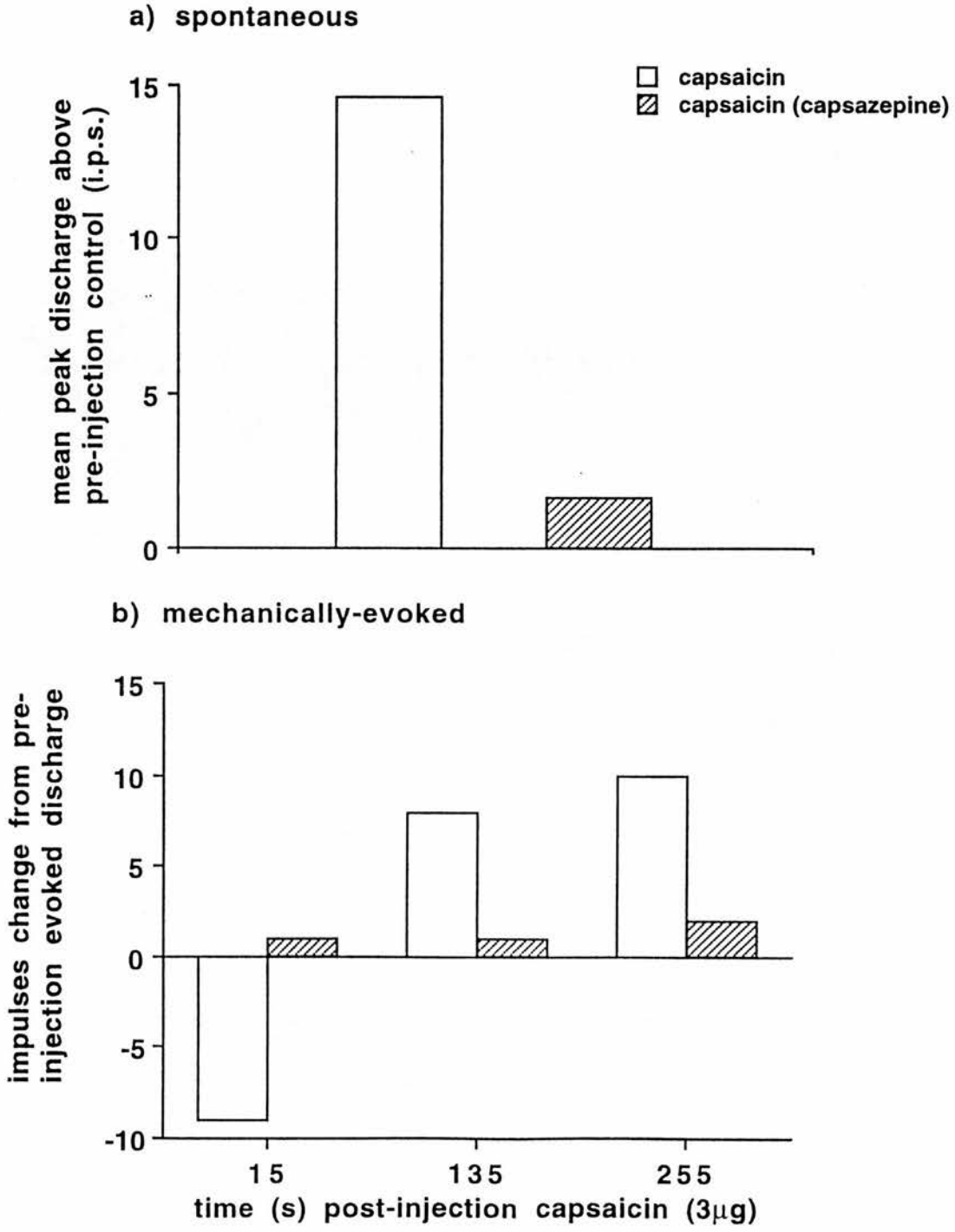



Figure 3.10 Effects of capsaicin (3μg, i.a.), before and after capsazepine (1mgkg$^{-1}$), on (a) spontaneous and (b) mechanically-evoked discharge from a single C-afferent fibre unit (conduction velocity 0.50ms$^{-1}$) in an arthritic (33 days post-adjuvant) ankle joint. The pre-injection spontaneous discharge, was 1.6 i.p.s pre-capsaicin and 1.9 i.p.s pre-capsaicin in the presence of capsazepine. The pre-injection mechanically-evoked discharge, was 32 impulses pre-capsaicin and 30 i.p.s pre-capsaicin in the presence of capsazepine. Note the capsaicin-induced desensitisation of the response to first mechanical stimulus post-capsaicin (b).

determine the effects of capsazepine itself on afferent neural discharge in these two units. Nevertheless, once neural discharge had returned to pre-capsazepine values a further injection of capsazepine ($1 mg kg^{-1}$, i.a.) was made with the assumption that the initial capsazepine was no longer active. This second injection of capsazepine had no effect on either spontaneous (3.5 ± 1.5 i.p.s. pre-capsazepine versus 3.6± 1.5 i.p.s. post-capsazepine) or mechanically-evoked (65 ± 35 impulses pre-capsazepine versus 66 ± 33 impulses post-capsazepine) discharge in the two units examined.

## 3.4 DISCUSSION

### 3.4.1 Gross pathology and behaviour of adjuvant-induced monoarthritic rats

The results of the present investigation confirm previous reports (Grubb et al., 1991; Donaldson et al., 1993) that a local injection (subdermally) of low dose Freunds adjuvant around a rat ankle joint induces an arthritis which remains confined to the injected joint, thus allowing the contralateral (uninjected) joint to be used as a within-animal control. Moreover, since the animals showed no other obvious systemic lesions, this monoarthritic model has advantages in terms of data interpretation, as any observed changes are more likely to be directly due to the inflammatory process itself. In contrast to these results, adjuvant-induced polyarthritis has a much more severe and widespread pathology; various joints are affected (particularly the hind limbs) and there is lesions in the eyes, ears, nose, skin, tail and genitals (Pearson & Wood, 1959; Ward & Jones, 1962; Pearson & Wood, 1963; Rainsford, 1982). In the current investigation, the localised nature of the adjuvant-induced arthritis is a result of the low dose (150μg) of adjuvant used (Ward & Jones, 1962; Donaldson et al., 1993) and the subdermal site of injection around the ankle joint (Billingham, 1983; Donaldson et al., 1993). Moreover, it has been shown by Donaldson et al. (1993) that by increasing the dose of adjuvant injected around the rat ankle joint to 250μg, a bilateral ankle joint arthritis can be induced. Thus, the method of injecting adjuvant locally around the ankle joint of the rat offers advantages in terms of experimental protocol as the degree of inflammation, whether uni- or bilateral, is simply determined by the dose of adjuvant injected.

In the present investigation two measurements (one quantitative, the other qualitative) indicative of mechanical hyperalgesia were made in adjuvant-induced monoarthritic rats. In the quantitative measurement, the pressure threshold for paw withdrawal in response to the application of direct pressure on the ankle joints was determined. The results from this test for mechanical hyperalgesia showed that the pressure required to evoke withdrawal of the limb was markedly reduced on the injected joint as compared to the contralateral uninjected joint (Figure 3.3). In agreement with this result, it has been shown that in polyarthritic rats direct pressure on the inflamed hind paw as in the Randall-Selitto test (Hirose & Jyoyama, 1975; Winter, 1979; Kayser & Guilbaud, 1981, 1983; Hara et al., 1984; Butler et al., 1985; Calvino & Le Bars, 1986) or by flexion of the arthritic ankle joint (Kuzuna & Kawai, 1975) causes hind limb withdrawal (mechanical hyperalgesia). Subjective measurements of foot placements (mobility) were made in order to obtain a measure of any mechanical hyperalgesia. Such a subjective measurement has the advantage that there is no need to either handle or restrain the rat, and thus the degree of stress is minimised. The significant reduction in the use of the chronically inflamed joint as shown by the subjective walking foot placement scores (Figure 3.4) probably reflects the reluctance of animals to experience the acute discomfort in the ankle joint associated with walking and load bearing.

It has been shown by Donaldson et al. (1993) that the gain in body weight of rats with a localised adjuvant-induced arthritis is not significantly different from that in untreated control rats. This result provides further evidence of the mild nature of

adjuvant-induced monoarthritis. In contrast, polyarthritic rats have been reported to show a profound loss of body weight (Pearson & Wood, 1959; Rainsford, 1982).

Following the injection of adjuvant around the rat ankle joint, the time course of the various parameters measured / assessed displayed four phases (Figures 3.1 - 3.4). For example, the limb circumference of the injected joint showed an initial increase (phase I: 1-3 days post-adjuvant) which then declined (phase II: 4-8 days post adjuvant) but then began to increase again (phase III: 8-18 days post-adjuvant) until a plateau was reached and maintained (phase IV: > 18 days post-adjuvant). These four phases are similar to those observed by investigators using adjuvant-induced monoarthritis (Birrell et al., 1990; Donaldson et al., 1993) or polyarthritis (see Rainsford, 1982; Billingham, 1983; Calvino et al., 1987). No studies to date have been done using the monoarthritic model to ascertain if these phases can be correlated to particular changes in the immune system. However, it has been shown in adjuvant-induced polyarthritis that different components in the immune system can be correlated with changes in inflammation and mechanical hyperalgesia. For example, it has been shown that lymphocytes play a major role in the early inflammation and hyperalgesia of adjuvant-induced arthritis (Burnstein & Waksman, 1964). The chronic phase of inflammation has been attributed to the increasing involvement of macrophages, monocytes and synoviocytes (Burnstein & Waksman, 1964). In the present study, there was a transient reduction in inflammation and hyperalgesia 4-8 days post adjuvant (phase II). This reduction, which requires investigation, may be explained by the gradual replacement of lymphocytes with macrophages and the involvement of synoviocytes and fibroblasts. In addition it has been shown that serum

albumin levels rise during this remission of acute inflammation (Billingham, 1983). Clearly, studies are required to determine the precise interactions between lymphocytes, macrophages and other cells (e.g. fibroblasts), the influence of inflammatory mediators (e.g. cytokines), which underlie each phase in adjuvant-induced poly- and mono-arthritis.

### 3.4.2 Histopathology of monoarthritic joints

Examination (by Mr H. F. Littlewood, Dr. P. Pilling and Mr. B. Reed, Department of Pathology, Glaxo Research & Development) of histological sections of the tibio-tarsal joints from rats with localised adjuvant-induced arthritis showed the presence of inflammatory cell infiltrate, synovial proliferation, fibroplasia, oedema and new bone formation (data not shown in this thesis). Such histopathogical features are substantially less severe than those associated with adjuvant-induced polyarthritis (Pearson & Wood, 1959). Moreover, bony ankylosis and joint deformation which occur in polyarthritis are not found in the monoarthritic model. Importantly, histological features of arthritis were localised to the injected joint with the contralateral uninjected tibio-tarsal joint showing a normal histological appearance (data not shown). Thus, the contralateral uninjected joint can be used as a within animal control.

### 3.4.3 *In-vivo* electrophysiology in normal and adjuvant-induced arthritic joints

The response characteristics of the high threshold slowly adapting articular mechanonociceptors found in the present investigation in rat ankle joints are similar to those reported previously in the rat ankle joint (Guilbaud et al., 1985; Grubb et al., 1988; Birrell et al., 1990; Grubb et al., 1991). The conduction velocities of all articular afferents were in the C-fibre range ($<1.5ms^{-1}$); there was no difference between conduction velocities obtained in normal joints and those from arthritic joints. In normal joints, mechanonociceptors exhibited little or no resting (spontaneous) discharge. In agreement with this result, little or no resting activity was seen in articular afferent units studied in normal rat ankle joints (Guilbaud et al., 1985; Grubb et al., 1988; Birrell et al., 1990;1993) or in normal cat knee joints (Coggeshall et al., 1983; Grigg et al., 1986; Schaible & Schmidt, 1985; 1988). In contrast to normal ankle joints, the results of the present investigation from articular mechanonociceptors in chronically inflamed (adjuvant-arthritic) joints showed greater (approximately 3 fold) levels of spontaneous discharge. Such inflammation-induced elevations in spontaneous C-fibre afferent discharge has also been reported in many preparations including chronically inflamed (adjuvant-induced) rat ankle joints (Guilbaud et al., 1985; Grubb et al., 1988; Birrell et al., 1990;1993), acutely inflamed (via intra-articular injection of kaolin and carrageenan) cat knee joints (Coggeshall et al., 1983) and in inflamed (induced by carrageenan or ultra-violet radiation) muscle (Berberich et al., 1988) and skin (Kocher et al., 1987; Szolcsányi, 1987).

Mechanical activation thresholds of mechanonociceptors in arthritic joints were generally lower than those in normal joints (Figure 3.5). Similarly, it has been reported by Schaible & Schmidt (1983a,b,; 1988) that nociceptors in the normal cat knee respond mainly to intense mechanical stimuli, to extreme flexion or extension and to joint rotation, whereas in contrast, there was a sensitisation to mechanical stimuli in the inflamed joint. Moreover, in these studies by Schaible & Schmidt, units in normal joints that were initially mechanoinsensitive became responsive to mechanical stimuli following inflammation of the joint. Therefore, it appears that during inflammation there is recruitment of mechanoreceptors which in normal tissues are unresponsive to mechanical stimuli. Such receptors have been termed 'silent or sleeping nociceptors' or more appropriately by Iggo (1988) as 'inflammation receptors'. The elevated resting discharge and mechanical sensitisation of mechanonociceptors seen in chronically-inflamed joints of the present investigation suggests that these altered neural discharges are important for the development of inflammatory pain and mechanical hyperalgesia. In subsequent sections of this thesis, the role of bradykinin, purines and adrenoceptor agents in altering the elevated neural discharges in chronically inflamed (adjuvant-induced arthritic) joints will be determined.

Electrophysiological recordings from rat articular (ankle joint) C-fibres obtained previously in this laboratory have established using peristimulus time histogram analysis that the majority (> 95%) of C-fibres from normal or arthritic ankle joints adapt slowly to steady mechanical indentation (see Guilbaud et al., 1985; Birrell, 1990; Grubb et al., 1991). A dynamic discharge during the application of the ramp

stimulus (indent) was followed by a static phase (slowly declining discharge) during the plateau of the mechanical indentation. On withdrawal of the probe there was an abrupt termination of the discharge (i.e. there was no after discharge). In the current investigation, the ability of articular C-fibres to adapt to mechanical stimuli was not studied in detail using peri-stimulus time histograms since previous investigators (McQueen, Grubb & Birrell) in this laboratory found it to be time consuming and did not show any change in response to the drugs being studied. Nevertheless, a visual analysis of the time interval between spikes was made routinely using a fast sweep of the oscilloscope face. Such visual analysis confirmed previous findings in this laboratory that C-fibres innervating rat ankle joints adapt slowly to mechanical indentation with dynamic and static phases; these two phases were still present even after the administration of the drugs used in this investigation (see Sections 3 - 7).

It is possible that the increase in spontaneous discharge and reduction of mechanical activation thresholds seen in single afferent units from arthritic joints were not due to a change in the excitability of mechanonociceptors but was secondary to changes in the mechanical properties of the inflamed joint. For example, in the arthritic joint there may have been more turgidity, tension and stiffness of the tissue such that pressure is applied more effectively to the individual mechanonociceptor. However, such inflammation-induced changes in tissue properties are unlikely to fully account for the large differences between activation thresholds between normal and arthritic joints (see Figure 3.5). It could also be argued that in the inflamed joint there is more connective tissue such that it becomes necessary to apply even more pressure to mechanically activate the mechanonociceptor. In effect this means that the activation

thresholds for units in arthritic joints are overestimates, and thus represents an even larger difference between normal and arthritic joints (see Figure 3.5).

It is impossible to say whether or not the chronic increase in mechanonociceptor discharge / reduction in mechanical activation threshold seen in arthritic joints of the rat leads to what is known as pain / hyperalgesia in man. However, elevations in mechanonociceptor discharge recorded from arthritic joints can be correlated with the results of behavioural studies by the application of Occams razor. For example, the reduction in latency of withdrawal of the arthritic rat hind limb following mechanical stimuli to the rat ankle joint (indicating nociception / hyperalgesia) can be related with the reduced mechanical activation thresholds seen in units recorded from arthritic joints. In humans, microneuropathy studies have correlated the 'pain' sensation of nociceptor C-fibre activation with its increase in neural discharge.

The elevations in receptor discharge seen in arthritic joints can also play a part in the inflammation process via the axon reflex (neurogenic inflammation). Essentially the axon-reflex involves antidromic stimulation of the primary afferent neurone; one branch of the neurone is regarded as the input side (via the nociceptor) and the other branch as the effector site where neuropeptides (e.g. substance P, neurokinin A and calcitonin-gene-related peptide) are released, following stimulation of the nociceptor, to act on target cells (e.g. mast cells) to generate an inflammatory response (see Foreman, 1987). Thus, via this axon-reflex-induced release of neuropeptides from the afferent neurone, elevations in receptor discharge are likely to contribute to the

changes seen in the behavioural studies such as enhanced limb circumference, increased inflammation and mechanical hyperalgesia.

### 3.4.4 Effects of capsaicin on mechanonociceptor discharge in normal and arthritic joints

Studies which examine the effects of drugs on sensory receptors using intra-arterial injection rely on access to the receptor terminals via the local microvasculature. One problem of such a route of drug administration is that if a sensory receptor is not excited then it is difficult to tell if this is a result of the drug being inactive or not reaching the receptor site. Thus, in order to test the responsiveness of sensory receptors a chemical known to cause sensory receptor excitation is required. Although injection of KCl causes sensory receptor excitation, it is unsuitable as its excitatory action is not selective for C-fibre afferents. The pungent constituent of hot peppers, capsaicin, has been used extensively as an agent which selectively excites C-fibre afferents (see Szolcsányi, 1993; Dray & Dickenson, 1993). In the current investigation, close arterial injection of capsaicin potently excited (high discharge, rapid onset and short response duration) articular mechanonociceptors. Therefore, capsaicin can be used as a suitable pharmacological probe to test the chemosensitivity of articular mechanonociceptors.

In the present investigation, capsaicin-induced increases in spontaneous and mechanically-evoked discharge were of greater magnitude and of longer duration in arthritic joints than in normal joints (Table 3.1, Figure 3.7 - 3.8). In agreement with these results, Birrell (1990) also reported an enhanced sensitivity of

mechanonociceptors to capsaicin in the arthritic ankle joint. Such an enhanced responsiveness to capsaicin in the inflamed joint may be a result of the presence of inflammatory mediators such as prostanoids or neuropeptides. Indeed, it has been shown in the neonatal rat spinal-cord preparation that $PGE_1$ and $PGE_2$ markedly potentiate capsaicin-induced ventral depolarisation via an effect on peripheral nerve endings (Yanagisawa et al., 1986). Moreover, capsaicin has been shown to release neuropeptides (e.g. substance P) from sensory terminals in the periphery, which in turn can also cause the release of mediators such as prostanoids or 5-HT (see Holzer, 1988).

Capsaicin-induced desensitisation has been extensively reported for C- fibre afferent nociceptors (see Dray & Dickenson, 1993; Szolcsányi, 1993). Such desensitisation was not observed in the present studies as only a low dose of capsaicin (1μg) was used in the experiments. Nevertheless, desensitisation of capsaicin-induced elevations in spontaneous and mechanically-evoked discharge have been shown in rat ankle joint mechanonociceptors when higher doses of capsaicin (10 - 30μg) are used (Birrell, 1990).

### 3.4.5 Preliminary studies of the effects of capsazepine

Recently, development of the novel capsaicin receptor antagonist, capsazepine, has provided convincing evidence for the existence of a capsaicin receptor (Dray & Dickenson, 1993; James et al., 1993; Walpole & Wrigglesworth, 1993). Capsazepine has been shown to be a highly selective antagonist of capsaicin-induced activation of

nociceptors both *in-vitro* (Bevan, 1991; Dickenson & Dray, 1991; Maggi et al., 1993) and *in-vivo* (Dickenson & Dray, 1991; Perkins & Campbell, 1992). In line with these studies, the current preliminary investigation showed that capsazepine antagonised the effects of capsaicin on both spontaneous and mechanically-evoked discharge of articular mechanonociceptors. Capsazepine itself appeared to have no effect on spontaneous or mechanically-evoked discharge. In agreement, other investigators also report a lack of direct effect of capsazepine on nociceptor activity (Dickenson & Dray, 1991; Perkins & Campbell, 1992). Thus, it is unlikely that capsaicin antagonists *per se* will be of use as analgesics.

## 3.5 SUMMARY

A monoarthritis was induced in the ankle joint of the rat by a localised injection of Freunds complete adjuvant. Such a model has scientific and ethical advantages over the polyarthritis model as only mild features of arthritis occur unlike the systemic disease seen in adjuvant-induced polyarthritis. Moreover, since arthritis is only present in the injected ipsilateral joint, this allows the uninjected contralateral joint to be used as a 'within-animal' control.

Electrophysiological recordings from C-fibre mechanonociceptors in adjuvant-arthritic joints showed higher levels of spontaneous discharge and lower mechanical activation thresholds than did those from normal joints.

Since close arterial injection of the selective C-fibre excitant, capsaicin, excited mechanonociceptors it can be used as a pharmacological probe to test the accessibility of chemosensitive C-fibre afferents via the blood supply. In preliminary studies, the novel selective capsaicin receptor antagonist, capsazepine, antagonised capsaicin-induced enhancements in spontaneous and mechanically-evoked discharge. Capsazepine itself was without effect on spontaneous or mechanically-evoked discharge.

## *SECTION 4*

# *EFFECTS OF INDOMETHACIN IN NORMAL AND ADJUVANT-ARTHRITIC RAT ANKLE JOINTS: ELECTROPHYSIOLOGICAL AND BEHAVIOURAL STUDIES.*

## 4.1 INTRODUCTION

In humans, disease states such as arthritis are characterised by pain, inflammation, and hyperalgesia, a state in which normally innocuous movement of limbs causes pain. In various animal behavioural models of nociception and hyperalgesia, sensitisation to mechanical stimuli (pressure, flexion / extension) have been observed. For example, behavioural investigations of rats with adjuvant-arthritis have established that the tibio-tarsal joints of the hind limbs of these animals become very sensitive (hyperalgesic) to mechanical stimuli (Kuzuna & Kawai, 1975; De Castro et al., 1981; Colpaert et al., 1982; Kayser & Guilbaud, 1983).

In electrophysiological studies, neural activity (spontaneous and mechanically-evoked) has been recorded from primary afferent C- and Aδ-fibres innervating normal and chronically arthritic rat ankle joints. The joint capsule of the rat ankle joint contains high threshold mechanoreceptors (Guilbaud et al., 1985) which are classified as type IV articular receptors having a nociceptive function (Wyke, 1981). In normal rat joints, such sensory articular receptors respond only to intense mechanical stimuli, and spontaneous discharge is either absent or very low (Guilbaud et al., 1985; Guilbaud & Iggo, 1985; Birrell et al., 1990; Grubb et al., 1991; McQueen et al., 1991). In contrast, articular mechanoreceptors in poly- or mono-arthritic ankle joints become sensitised to mechanical stimuli, and afferent fibres show higher levels of spontaneous discharge as compared to normal joints (Guilbaud & Iggo, 1985; Guilbaud et al., 1985; Birrell et al., 1990; Grubb et al., 1991; McQueen et al., 1991; see also Section 3). Raised resting (spontaneous) discharge and enhanced discharge to

mechanical stimuli from articular mechanonociceptors have also been demonstrated in the cat following inflammation of the knee joint (Coggeshall et al., 1983; Grigg et al., 1986; Schaible & Schmidt, 1985; Schaible & Schmidt, 1988).

One of the most commonly prescribed group of agents for treating the pain, hyperalgesia and inflammation found in inflammatory states such as rheumatoid arthritis are the non-steroidal anti-inflammatory drugs (NSAIDs). The various NSAIDs available vary considerably in their ability to produce analgesia and in their anti-inflammatory properties (Rang & Dale, 1991). Indomethacin is a NSAID which has both analgesic and anti-inflammatory actions (Rang & Dale, 1991), and is used for the treatment of rheumatic disorders (Hellberg, 1981; Seideman & Eriksson, 1988; Seideman & Melander, 1988).

The aims of the present investigation were two-fold. Firstly, an electrophysiological study to determine the ability of indomethacin to modify neural discharge (spontaneous and mechanically-evoked) from fine articular afferents innervating normal joints, and in joints with Freunds adjuvant-induced chronic monoarthritis. Secondly, a behavioural study, in order to examine the effects of indomethacin on parameters of hyperalgesia and inflammation in rats with an established localised arthritis induced by Freunds adjuvant.

## 4.2 MATERIALS & METHODS

### 4.2.1 Electrophysiological studies

The *in-vivo* preparation, neural recording, off-line analysis and statistical analysis are described in detail in Section 2. In brief, male Wistar rats (normal and adjuvant-arthritic) were anaesthetised with urethane and cannulations performed of the trachea, right carotid artery (blood pressure monitoring) and right femoral artery (retrograde cannulation for close intra-arterial bolus injections of drugs into the left limb). The medial aspect of the left ankle joint was exposed and nerve fibres were isolated from the PACR nerve. C-fibre afferent discharge (spontaneous and mechanically-evoked) from articular mechanonociceptors was recorded extracellularly, using bipolar platinum-iridium electrodes.

**Protocol**

A single close intra-arterial injection of indomethacin ($10mgkg^{-1}$) was made in either normal rats or in rats with chronic adjuvant-induced monoarthritis. This dose of $10mgkg^{-1}$ is consistent with that used once daily by other investigators in the rat ($10mgkg^{-1}$, i.v.: Ferreira et al., 1978; $8mgkg^{-1}$ i.v.: Jurna & Brune, 1990; $10mgkg^{-1}$ s.c.: Novo et al., 1992).

**Data analysis**

Mechanically-evoked neural discharge was quantified as the number of impulses per stimulus. The reduction in spontaneous discharge induced by indomethacin was quantified by averaging discharge over successive 100s time periods (i.e. between

mechanical indentations). The control period of spontaneous discharge was defined as the 60s period immediately prior to the injection of indomethacin.

### 4.2.2 Behavioural studies

The effects of injecting indomethacin (bolus injection i.p. once daily, $0.5mgkg^{-1}$) in rats with established (14 days post-adjuvant) localised Freunds adjuvant-induced arthritis of the left ankle joint was examined. Rats were subjected to a series of quantitative tests involving the measurement of ankle joint circumferences, and foot withdrawal following the application of pressure to the left (adjuvant-injected) and right (un-injected) ankle joints. Qualitative tests were also performed to obtain measurements of inflammation and mobility of the left (adjuvant-injected) and right (un-injected) ankle joints. The various measurements were made approximately 30min after drug injections. Rat body weight was used as a general indicator of animal health. All measurements were made blind to drug treatment by the same experienced investigator. The various quantitative and qualitative tests and the statistical analysis are described in detail in Section 2.2.1

## 4.3 RESULTS

### 4.3.1 *In-vivo* electrophysiology in normal and adjuvant-arthritic rat ankle joints

The effects of indomethacin, were examined in six units (six experiments) from normal joints and in six units (six experiments) from adjuvant-arthritic (21 ± 2 days post-adjuvant) joints. The mean afferent conduction velocity was in the C-fibre range for units examined either from normal (0.66 ± 0.15; range: 0.22 - 1.30) or arthritic (0.70 ± 0.13; range: 0.20 - 1.20$ms^{-1}$ ) joints. There was no significant difference between the afferent conduction velocities of units in normal joints with those in arthritic joints ($P>0.05$, Mann Whitney U-test). Before the addition of any drugs, all units showed resting (spontaneous) discharge, although these were significantly greater ($P<0.05$, Mann Whitney U-test) in units from arthritic joints (4.58 ± 1.07 i.p.s.; range:1.4 - 8.0 i.p.s.) than in those from normal joints (1.03 ± 0.58 i.p.s.; range: 0.5 - 1.3 i.p.s.) . All the units examined were excited by close intra-arterial injection of capsaicin (1 - 3μg).

#### 4.3.1.1 Effects of indomethacin on mechanonociceptor discharge from arthritic joints

##### 4.3.1.1.1 Effects of indomethacin on spontaneous mechanonociceptor discharge

Compared to pre-injection discharge levels, injection of indomethacin (10$mgkg^{-1}$, i.a.) caused a significant reduction in ongoing (spontaneous) discharge in all six units (six experiments) examined (see Figure 4.1 for a typical indomethacin-induced

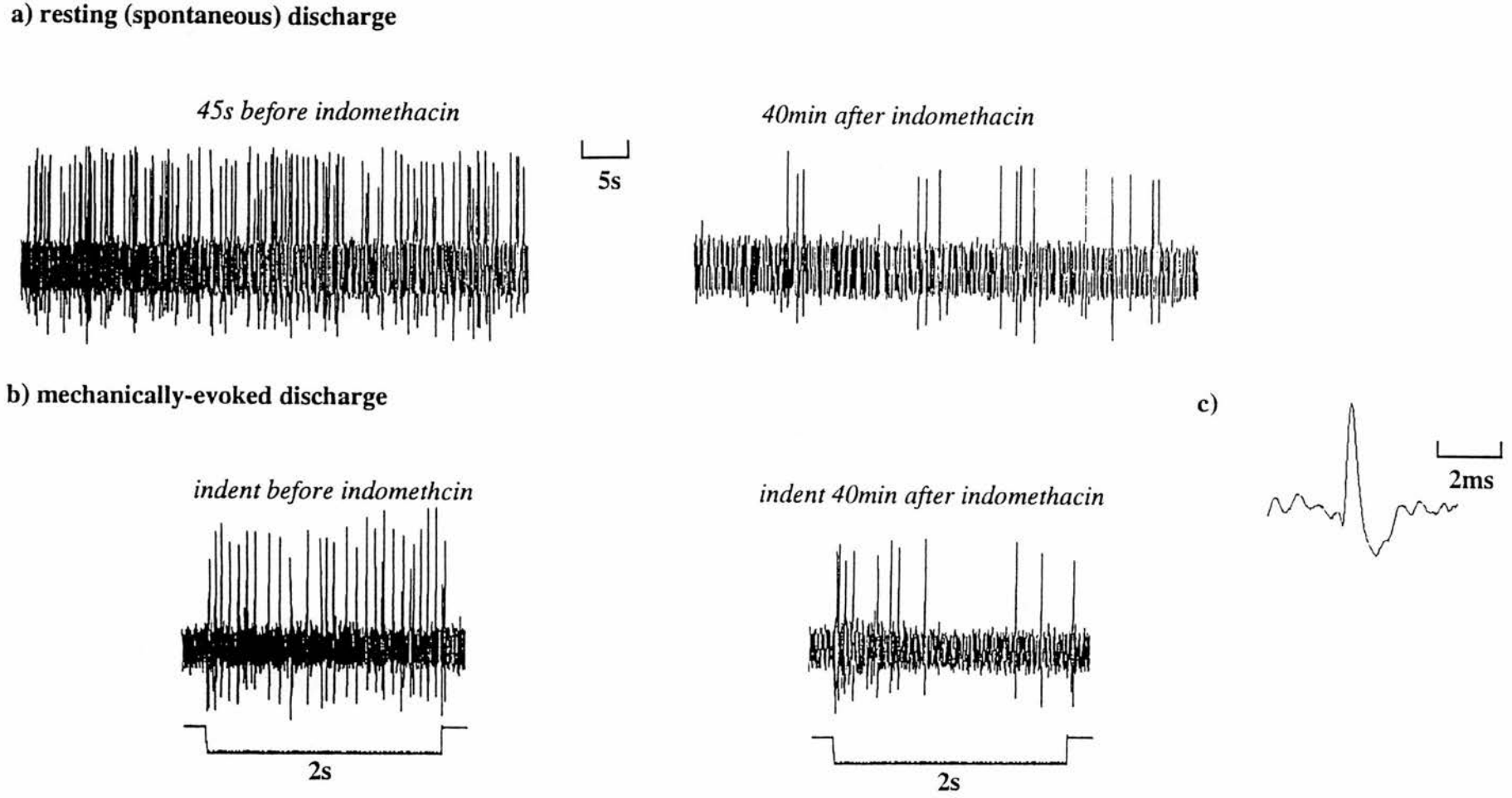



Figure 4.1 Neurograms showing indomethacin (10mgkg$^{-1}$, i.a.)-induced reductions in (a) spontaneous and (b) mechanically-evoked discharge from a single articular mechanonociceptor with an afferent conduction velocity of 0.7ms$^{-1}$. (a) The control spontaneous discharge was 2.5 i.p.s., and 0.2 i.p.s. 40min after the injection of indomethacin. (b) The waveform of the mechanical indentor is shown below the neurogram. The control mechanically-evoked discharge was 24 spikes, which was reduced to 11spikes 40min after injection of indomethacin. (c) A fast oscilloscope sweep of the unit.

reduction and pooled data in Figure 4.2). As reflected in the standard errors in Figure 4.2, the indomethacin-induced reduction in spontaneous discharge varied between experiments. This variability is a consequence of pooling data that have a wide range of basal values, coupled with the observation that individual mechanoreceptors differed in their responsiveness to indomethacin: all showed depression of spontaneous discharge, but the magnitude of the reduction and the time course was variable. Consequently, in order to eliminate inter-experiment variability attributable to differing basal values, the data were normalised as a percentage of the pre-injection control values. Plotting the data in this way shows that indomethacin caused similar reductions (Figure 4.2). In comparison with vehicle injections, indomethacin caused a significant reduction in spontaneous discharge (Figure 4.3).

The indomethacin induced reduction in spontaneous discharge had a mean latency to onset of 2.7 ± 0.67min (range: 2 - 4min). After injection of indomethacin, spontaneous discharge reached a mean minimum value to 24% (range 0 - 50%) of the pre-injection control level (100%), after a delay of 15 ± 4min (range: 12 - 24min).

#### 4.3.1.1.2 Effects of indomethacin on mechanically-evoked discharge

Injection of indomethacin (10mgkg$^{-1}$, i.a.) caused a significant reduction in mechanically-evoked discharge in all six units (six experiments) examined (see Figure 4.1 for a typical indomethacin-induced reduction). As described previously (Section 4.2.1.1.1), in order to eliminate inter-experiment variability attributable to differing basal values (Figure 4.4), the data were normalised as a percentage of the

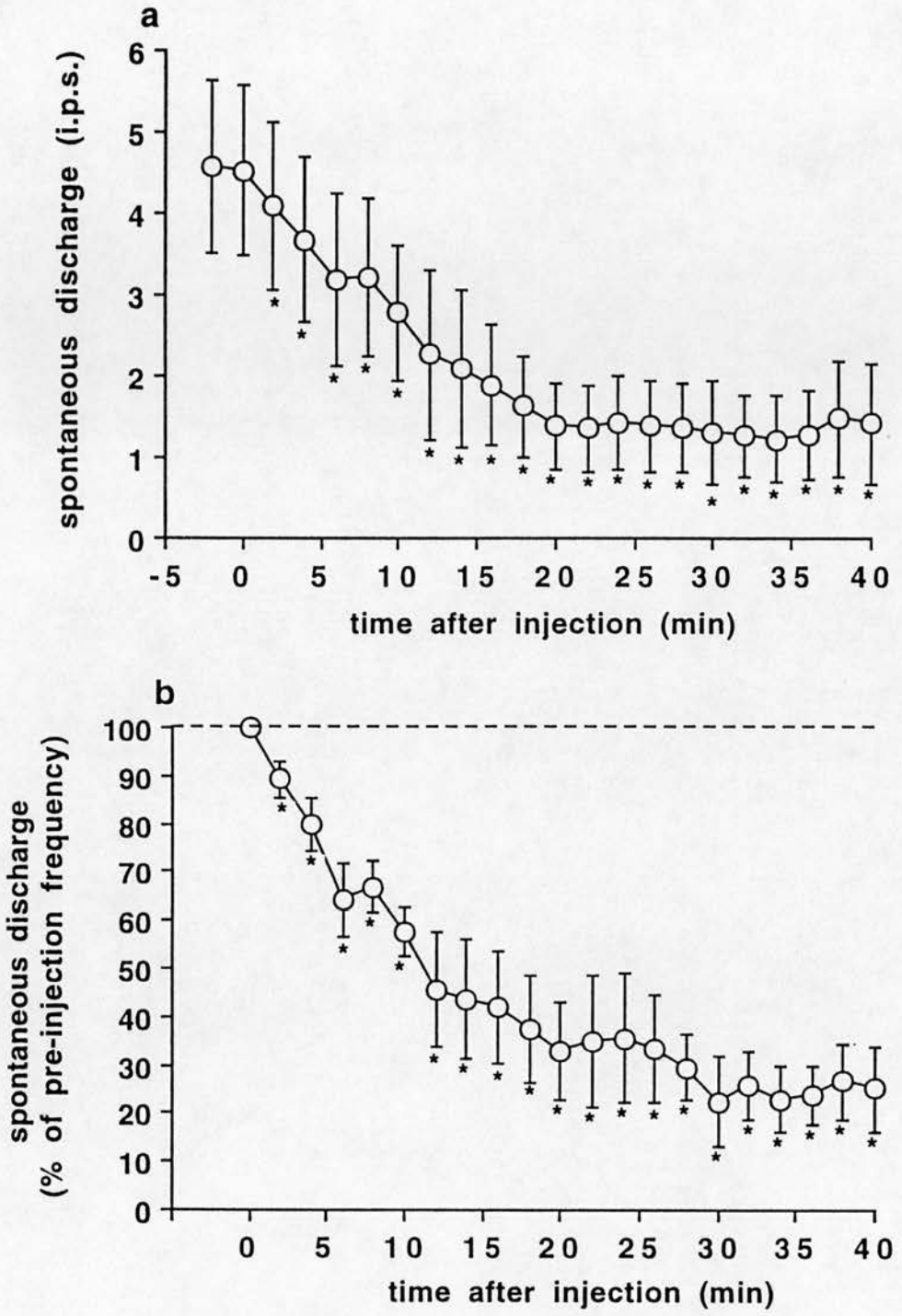



Figure 4.2 Effects of injecting indomethacin (10mgkg$^{-1}$, i.a.) on spontaneous discharge from articular mechanonociceptors in arthritic ankle joints. Basal (a) and normalised (b, dashed line represents the pre-injection response:100%) values are shown. Each point is the mean ± s.e.mean from n=6 units (6 experiments). * $P<0.05$, Wilcoxon test, versus pre-injection control discharge.

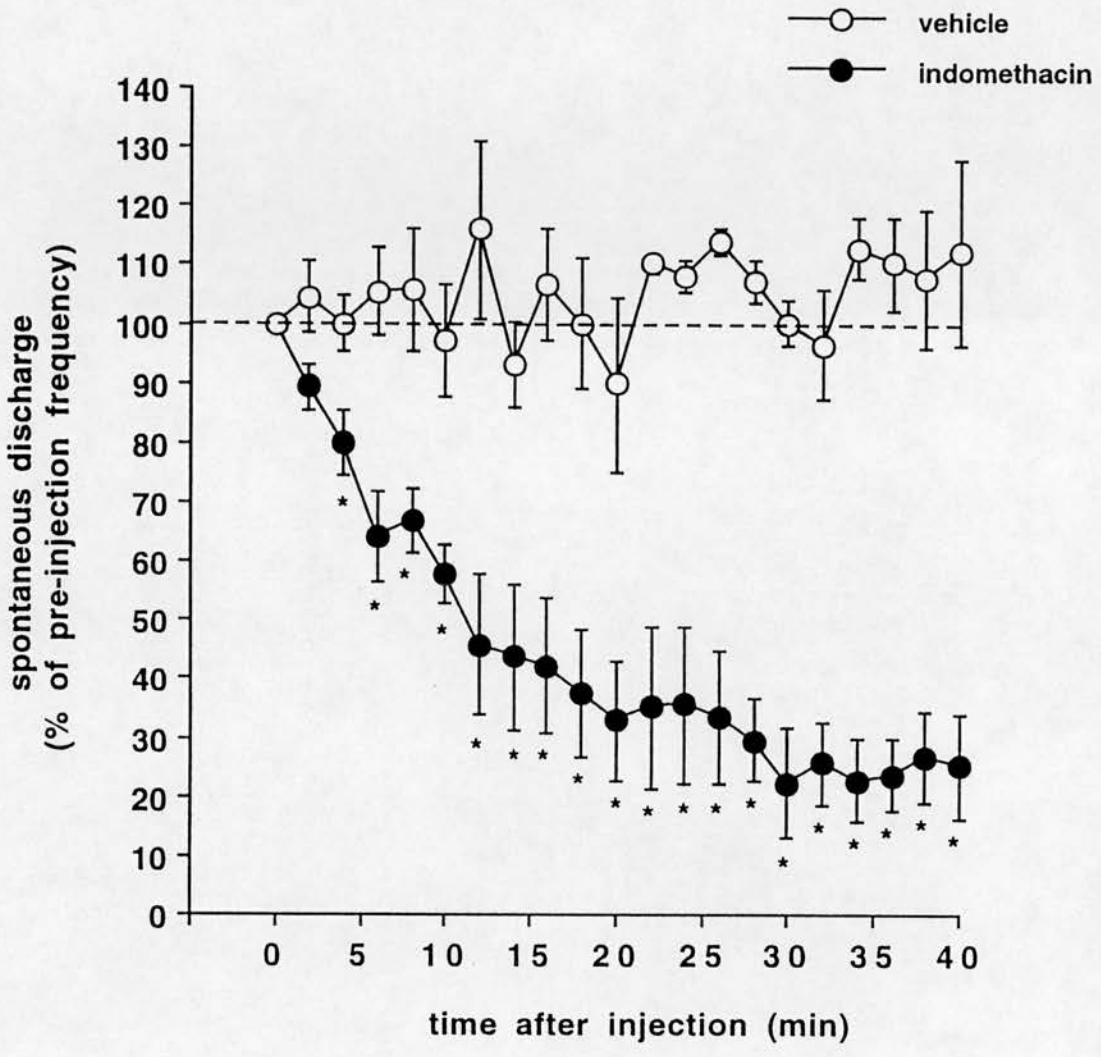



Figure 4.3 Normalised data showing the effects of injecting indomethacin (10mgkg$^{-1}$ i.a. 0.3 - 0.5ml, n=6 units: 6 experiments) or vehicle (0.5ml, n=6 units: 4 experiments) on spontaneous discharge from articular mechanonociceptors in arthritic ankle joints. The dashed line represents the pre-injection response (100%). Each point is the mean ± s.e.mean. * $P<0.05$, Mann Whitney U-test, versus vehicle.

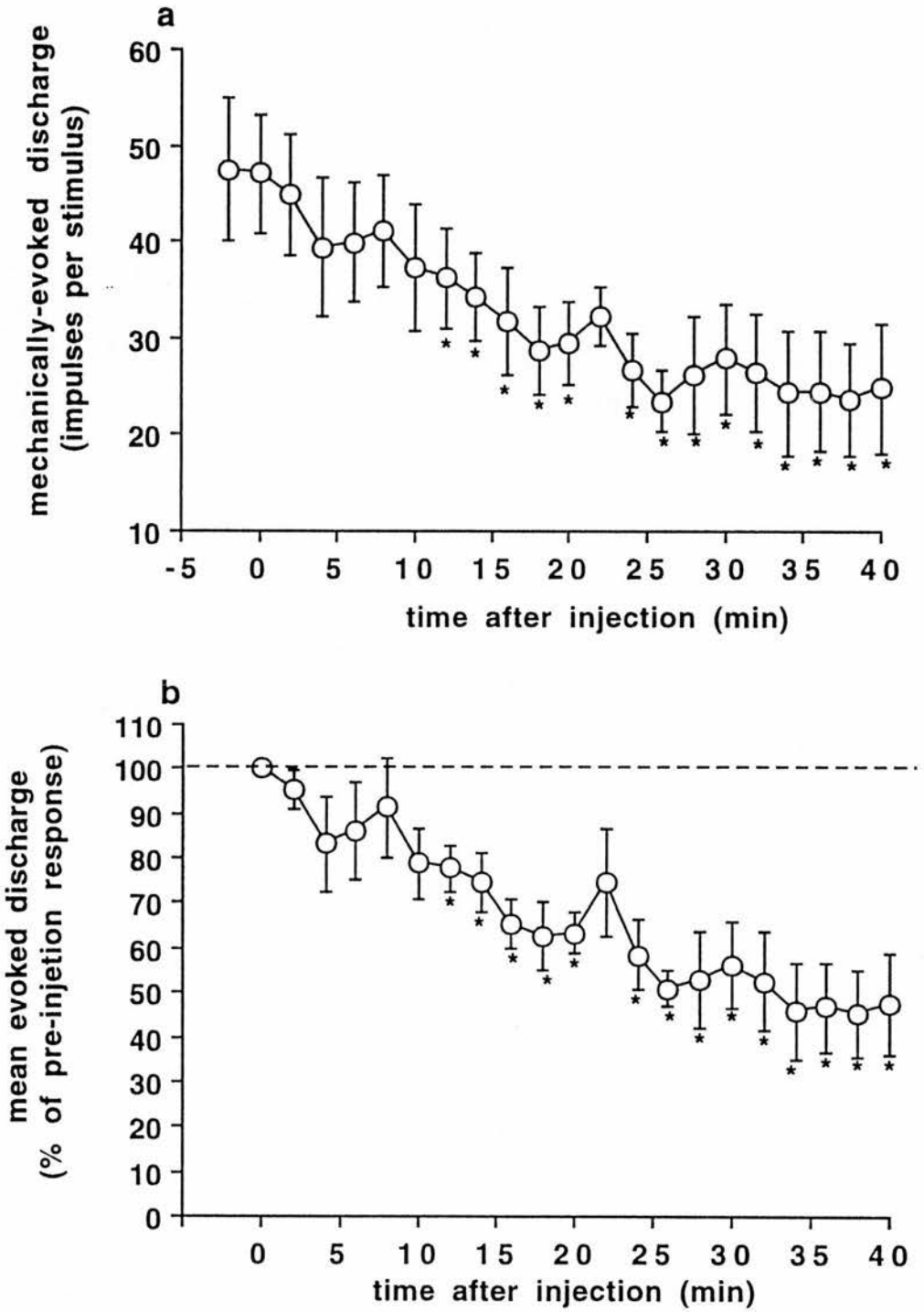



Figure 4.4 Effects of injecting indomethacin (10mgkg$^{-1}$, i.a.) on the discharge evoked by mechanical stimulation of articular mechanonociceptors in arthritic ankle joints. Basal (a) and normalised (b, dashed line represents the pre-injection response:100%) values are shown. Each point is the mean ± s.e.mean from n=6 units (6 experiments). * $P<0.05$, Wilcoxon test, versus pre-injection control discharge.

pre-injection control values. Plotting the data in this way shows that indomethacin caused similar reductions in mechanically-evoked discharge (Figure 4.4). In comparison with vehicle injections, indomethacin significantly produced a decrease in the responsiveness of the standard mechanical stimulus (Figure 4.5)

The indomethacin-induced reduction in the responsiveness to the standard mechanical stimulus had a mean latency to onset of 5 ± 2min (range: 2 - 12min). Mechanoreceptor responsiveness reached a mean minimal value to 45% (range 0 - 68%) of the pre-injection control following a mean delay period of 32 ± 4min (range: 26 - 36min).

#### 4.3.1.1.3 Duration of indomethacin-induced depression in mechanonociceptor discharge

As the preparations were used for other experiments between 42-44min post-injection of indomethacin (see Section 7.3.6), the time in which spontaneous and mechanically-evoked discharge returned to pre-injection control discharge values could not be determined. Nevertheless, spontaneous and mechanically-evoked discharge 90-100min post-injection of indomethacin (bradykinin 1-30μg, i.a. was injected 40-70min after indomethacin) were depressed by 75 ± 8% and 51 ± 7%, respectively, in six units (six experiments), indicating that indomethacin has a long duration of action.

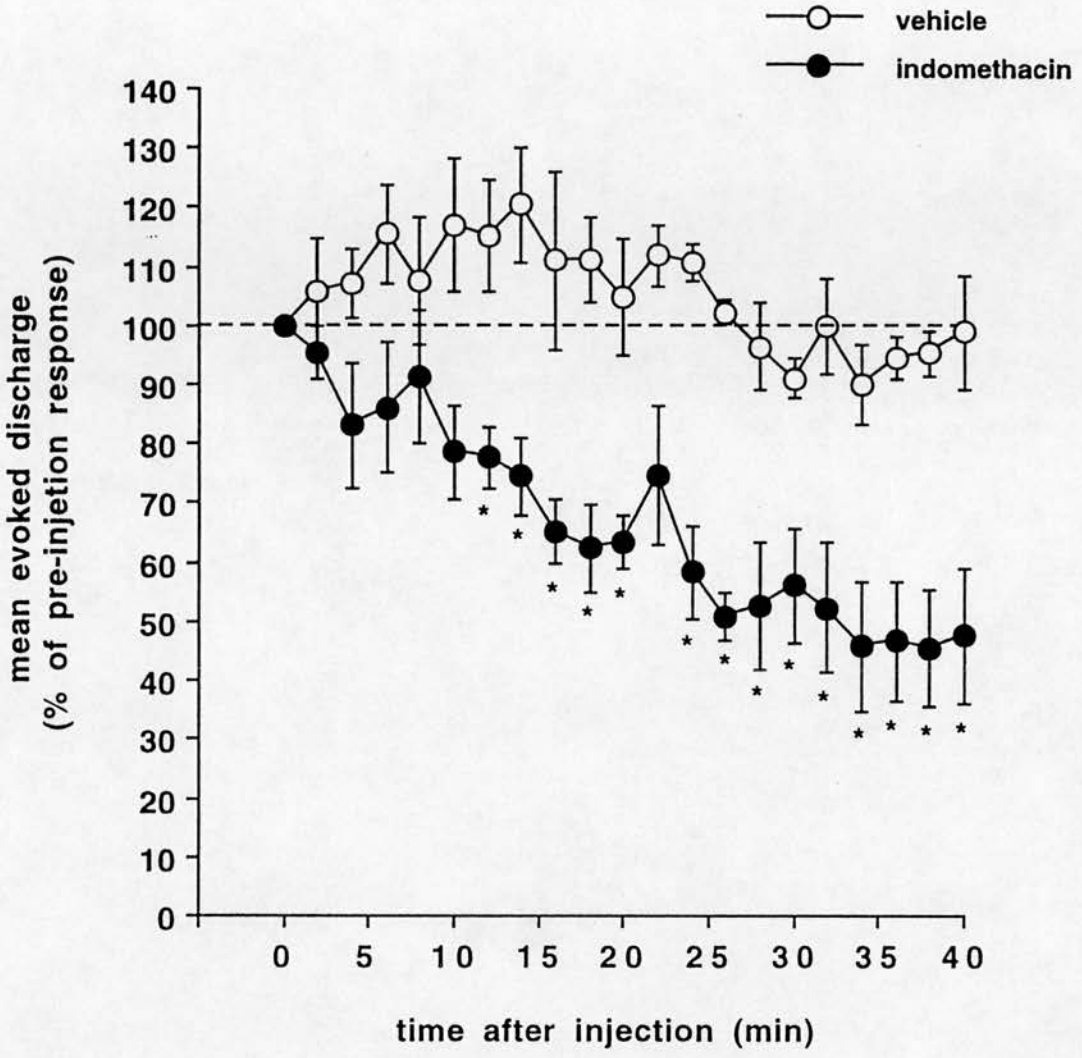



Figure 4.5 Normalised data showing the effects of injecting indomethacin (10mgkg$^{-1}$ i.a. 0.3 - 0.5ml, n=6 units: 6 experiments) or vehicle (0.5ml, n=6 units: 4 experiments) on the discharge evoked by mechanical stimulation of articular mechanonociceptors in arthritic ankle joints. The dashed line represents the pre-injection response (100%). Each point is the mean ± s.e.mean. * P<0.05, Mann Whitney U-test, versus vehicle.

#### 4.3.1.2 Effects of indomethacin on mechanonociceptor discharge from normal joints

Compared to pre-injection discharge levels, indomethacin ($10mgkg^{-1}$) had no effect on either spontaneous discharge (Figure 4.6) or on the responsiveness to the standard mechanical stimulus (Figure 4.7) in any of the six units (six experiments) examined.

### 4.3.2 Influence of indomethacin on an established adjuvant-induced arthritis in the rat

The subdermal injection of Freunds adjuvant (0.15mg: 0.15ml of $1mgml^{-1}$ solution) around the left ankle joint resulted in a swelling, hyperalgesia and inflammation of the joint which became established around 10 - 14 days post-injection of adjuvant (see Section 3). In indomethacin-treated rats, there were reductions in the circumference (Figure 4.8) and inflammation (Figure 4.9) of the arthritic ankle joint, and in the mobility of the left hind limb (Figure 4.10) as compared with vehicle-treated rats. Indomethacin also caused a significant increase in the pressure required to cause withdrawal of the adjuvant-arthritic left hind limb (Figure 4.11). Indomethacin had no significant effect ($P>0.05$) on circumference, and on inflammation, mobility and paw withdrawal pressure scores, of the contralateral (un-injected) ankle joint (data not shown). Indomethacin-treated rats showed significant weight gains as compared with vehicle-treated rats (Figure 4.12).

In general, it was found that the effects of indomethacin on the various parameters measured outlasted the period of indomethacin treatment (Figures 4.8 - 4.12).

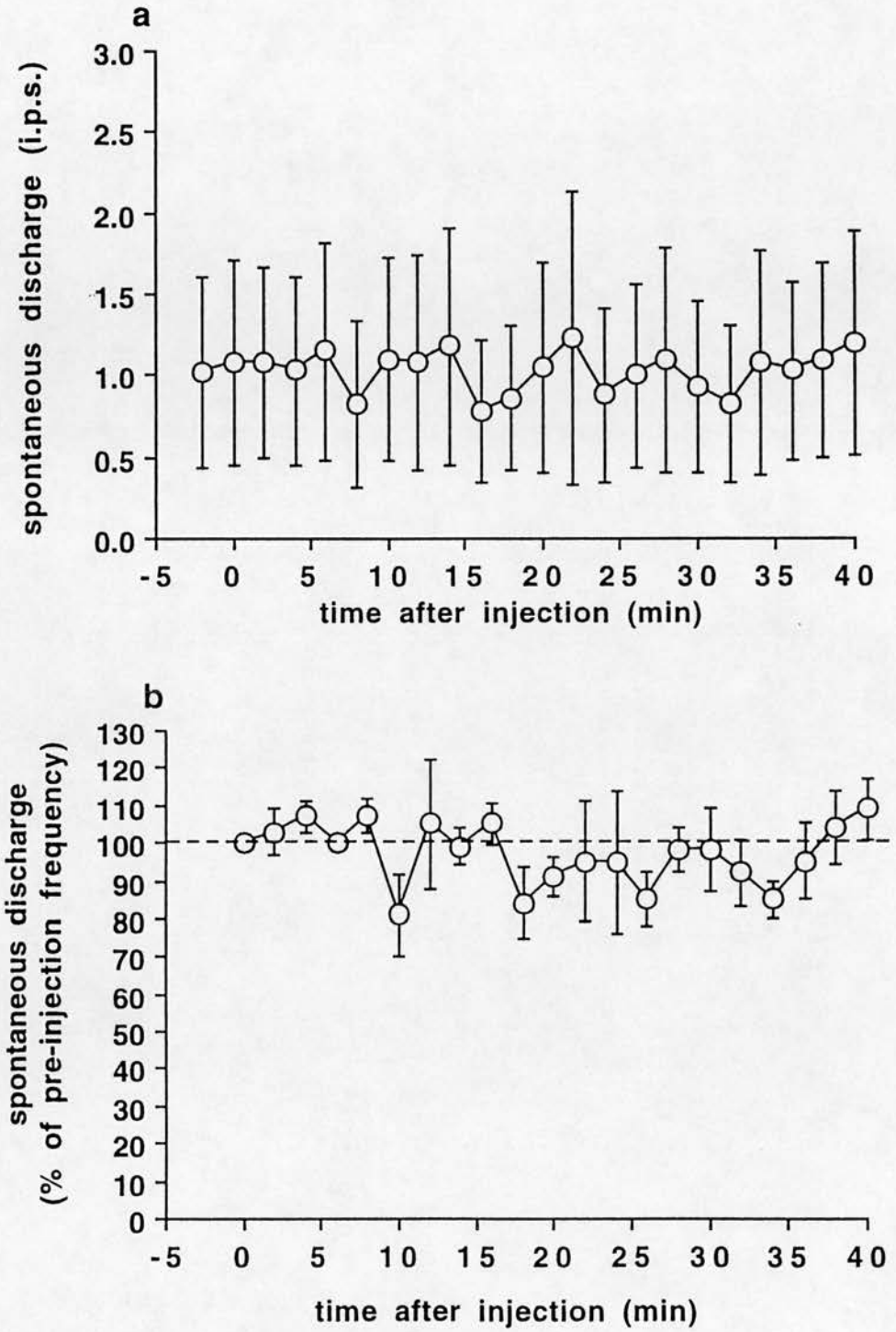



Figure 4.6 Effects of injecting indomethacin (10mgkg$^{-1}$, i.a.) on spontaneous discharge from articular mechanonociceptors in normal ankle joints. Basal (a) and normalised (b, dashed line represents the pre-injection response:100%) values are shown. Each point is the mean ± s.e.mean from n=6 units (6 experiments). P>0.05, Wilcoxon test, versus pre-injection control discharge.

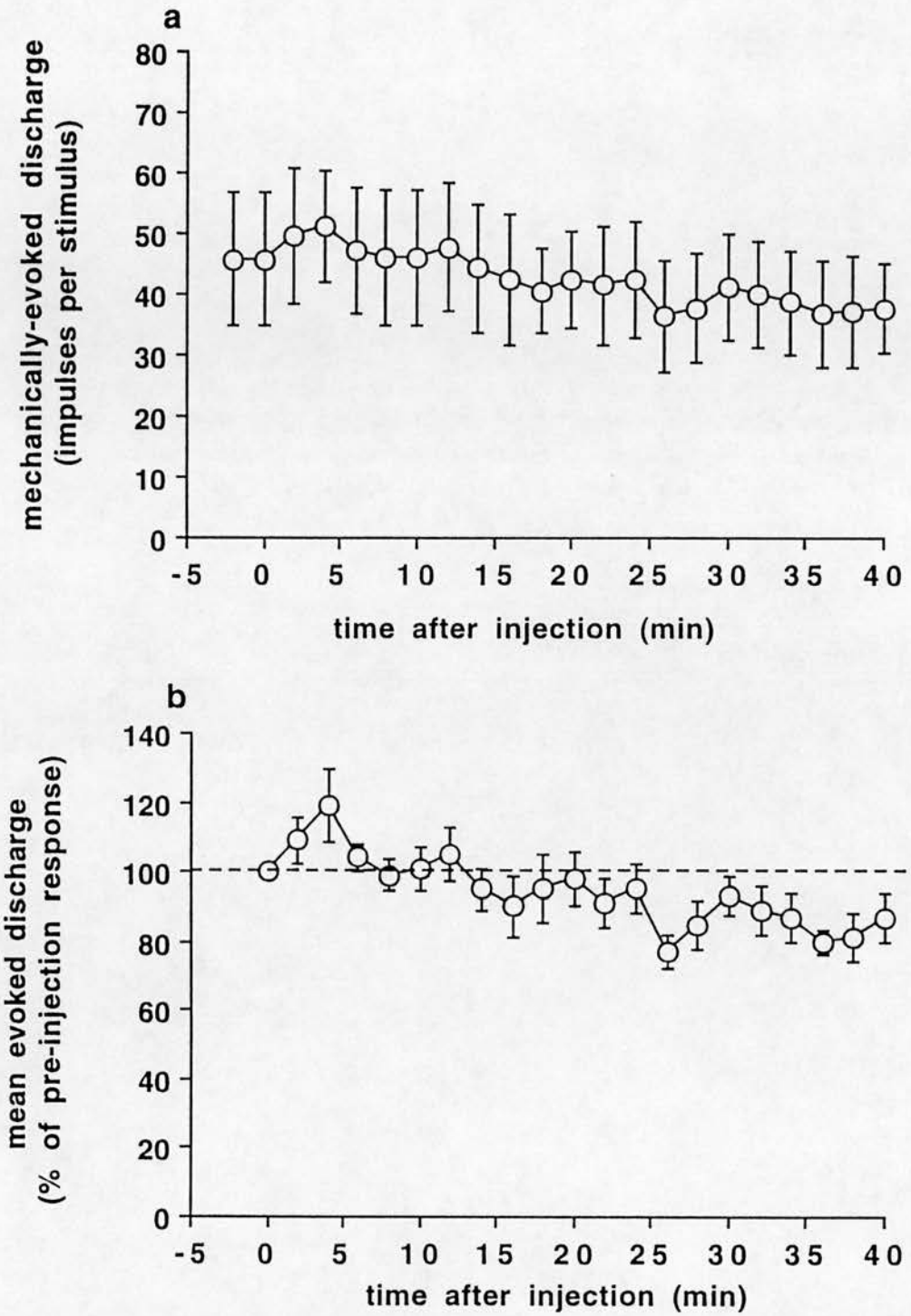



Figure 4.7 Effects of injecting indomethacin (10mgkg$^{-1}$, i.a.) on the discharge evoked by mechanical stimulation of articular mechanonociceptors in normal ankle joints. Basal (a) and normalised (b, dashed line represents the pre-injection response:100%) values are shown. Each point is the mean ± s.e.mean from n=6 units (6 experiments). P>0.05, Wilcoxon test, versus pre-injection control discharge.

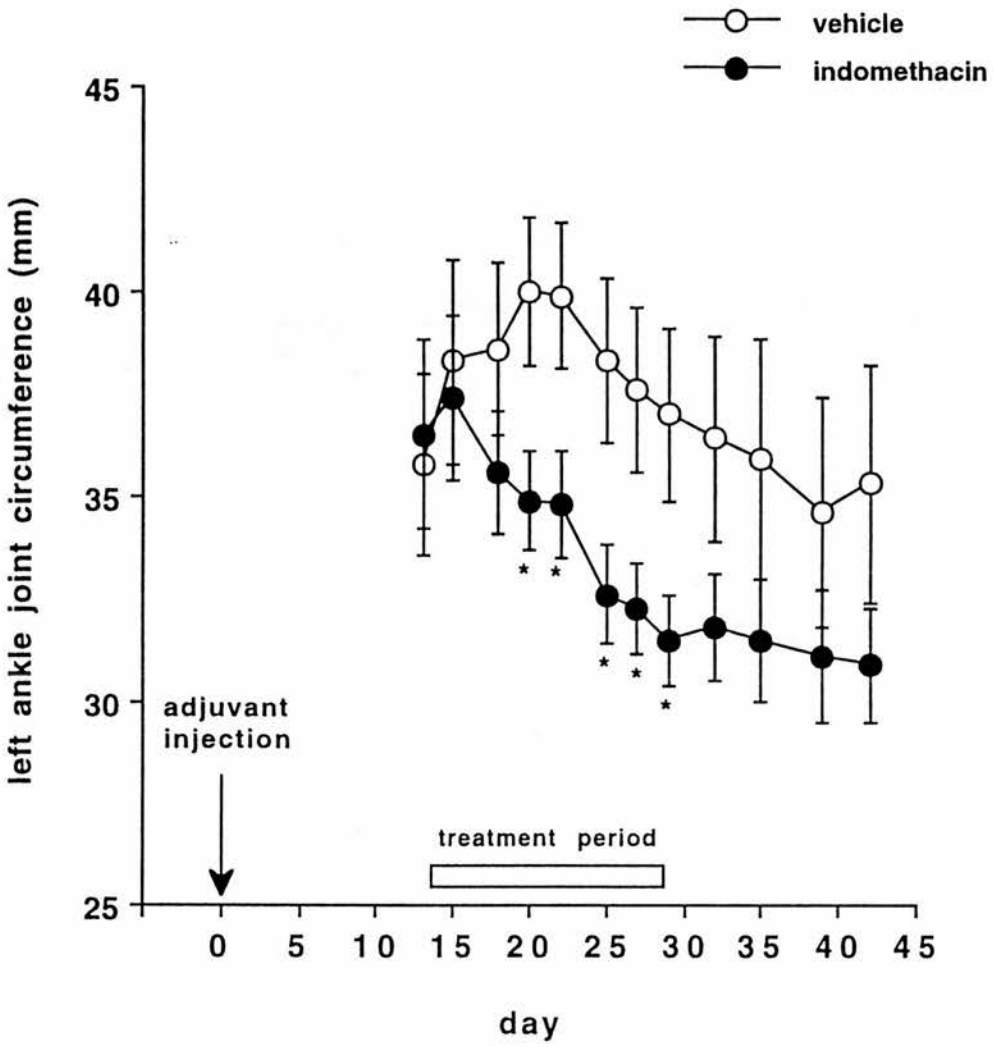



Figure 4.8 Effects of vehicle or indomethacin ($0.5 mgkg^{-1}$, i.p.) on the circumference of adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: $P<0.05$, ANOVA days 20-29.

* $P<0.05$, Mann Whitney U-test, versus vehicle.

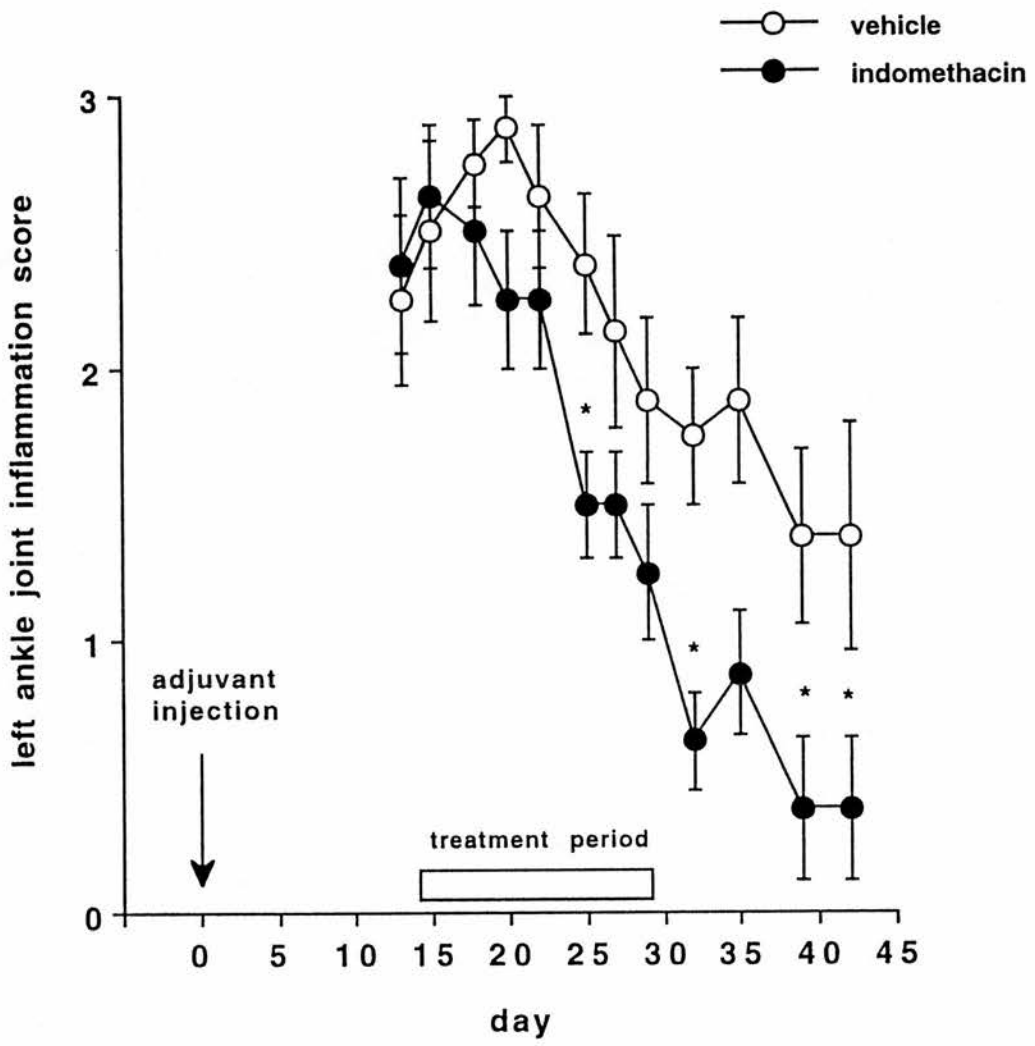



Figure 4.9 Effects of vehicle or indomethacin (0.5mgkg$^{-1}$, i.p.) on inflammation scores of adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: * P<0.05, Mann Whitney U-test, versus vehicle.

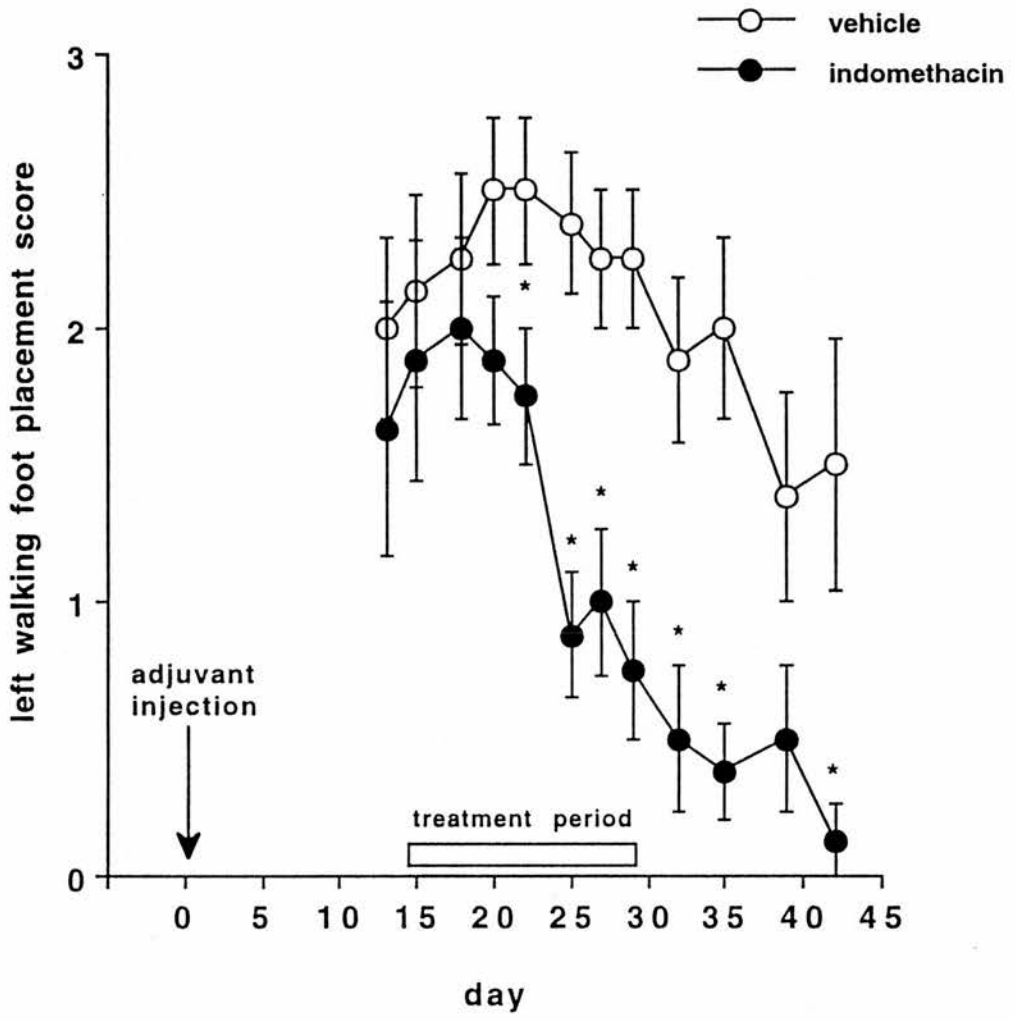



Figure 4.10 Effects of vehicle or indomethacin ($0.5mgkg^{-1}$ ,i.p.) on left walking foot placement (mobility) scores in rats with adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: * $P<0.05$, Mann Whitney U-test, versus vehicle.

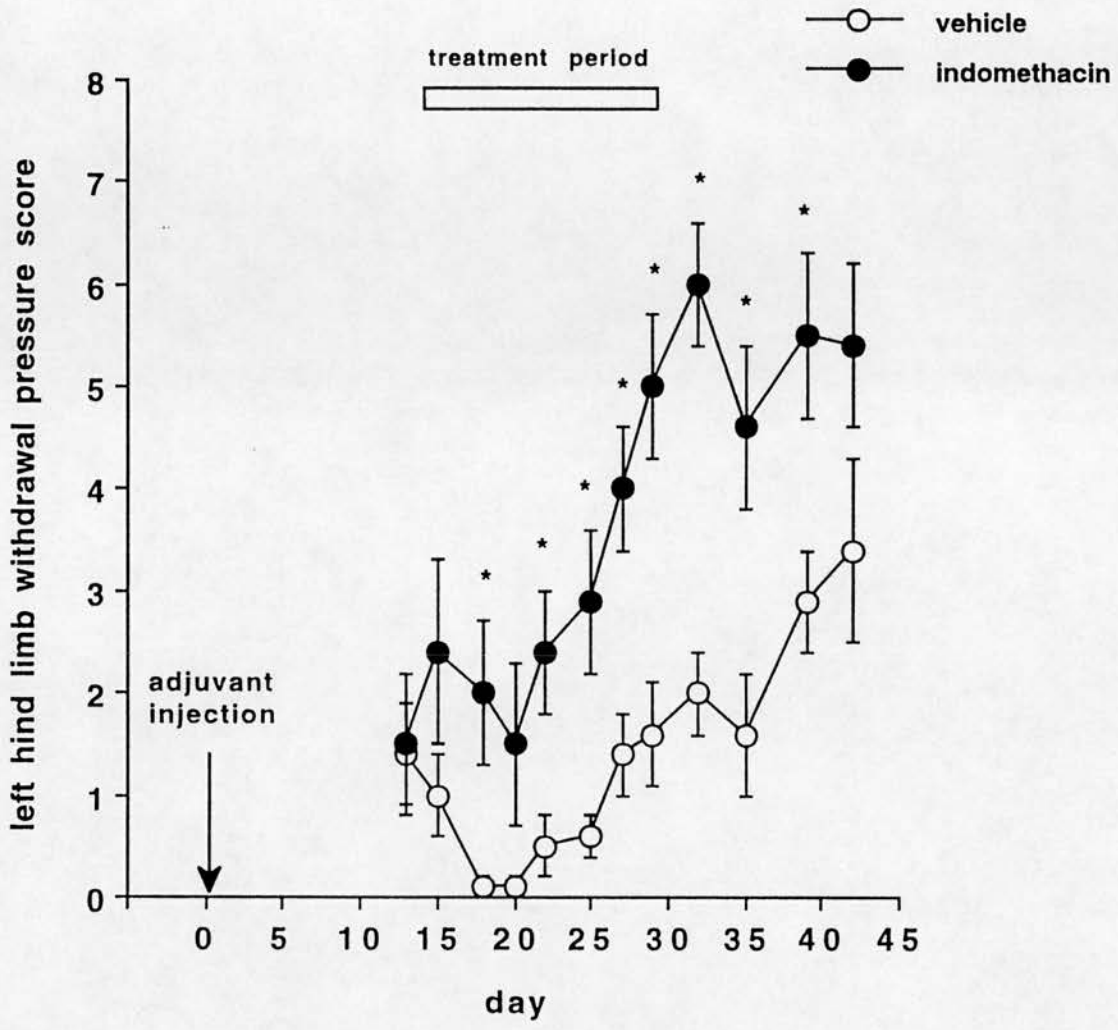



Figure 4.11 Effects of vehicle or indomethacin ($0.5mgkg^{-1}$, i.p.) on the pressure-evoked withdrawal thresholds of adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: $P<0.05$ ANOVA days 14-29.* $P<0.05$, Mann Whitney U-test, versus vehicle.

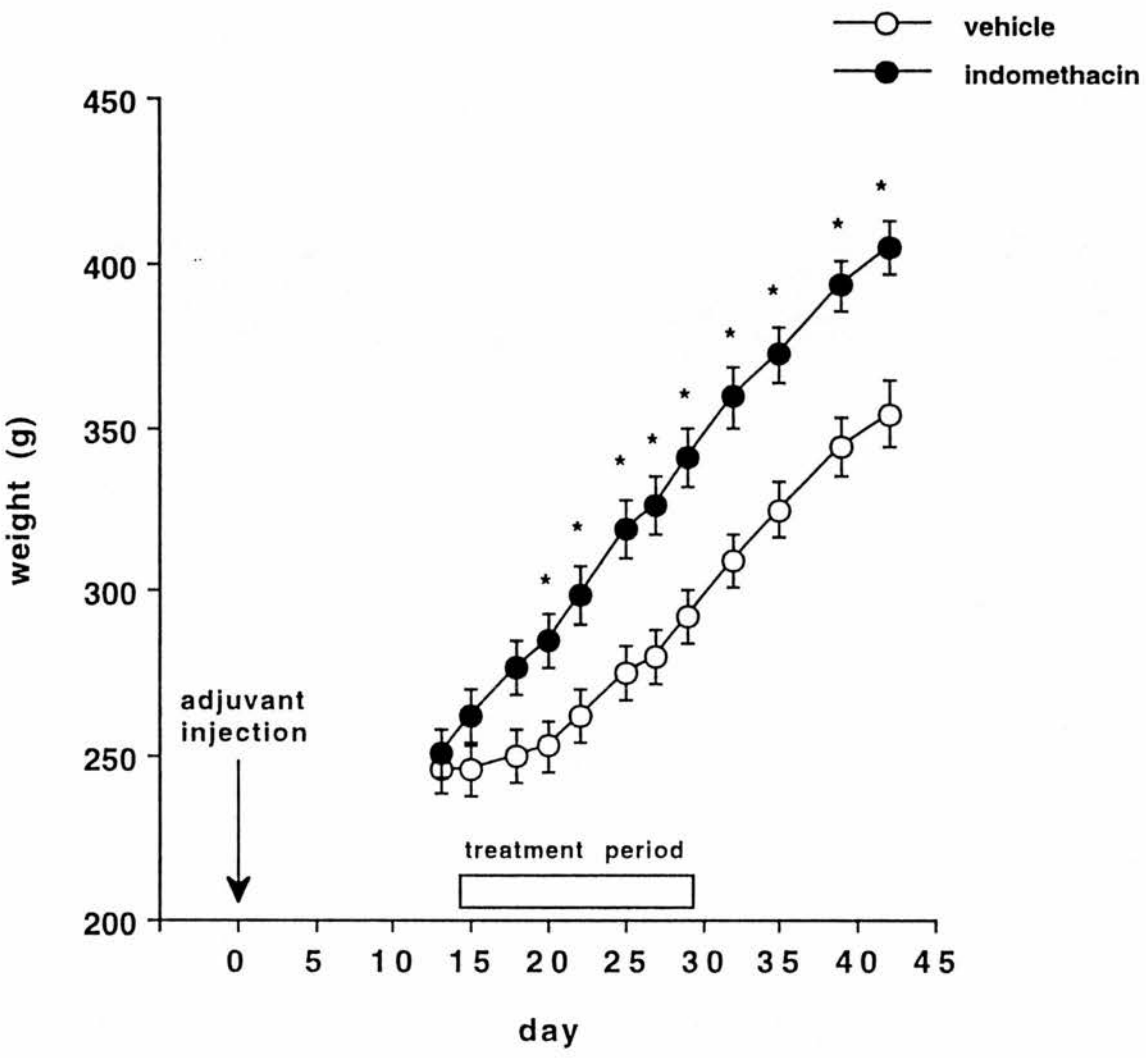



Figure 4.12 Effects of vehicle or indomethacin ($0.5 mgkg^{-1}$, i.p.) on weight of rats with adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: $P<0.05$ ANOVA days 14-29. * $P<0.05$, Mann Whitney U-test, versus vehicle.

## 4.4 DISCUSSION

### 4.4.1 Effects of indomethacin in electrophysiological studies

The results of the present investigation have shown that neural discharge (spontaneous and mechanically-evoked) from C-fibre afferents innervating adjuvant-arthritic rat ankle joints was reduced by the NSAID, indomethacin. These findings are in general agreement with similar electrophysiological investigations using adjuvant arthritic (mono- and poly-arthritic) rat ankle joint preparations where the NSAIDs, lysine acetylsalicylate and paracetamol, caused depressions in spontaneous and mechanically-evoked discharge (Guilbaud & Iggo, 1985; Birrell, 1990; Grubb et al., 1991; McQueen et al., 1991). However, whereas McQueen et al. (1991) reported that lysine acetylsalicylate ($100mgkg^{-1}$; equivalent to $50mgkg^{-1}$ aspirin) or paracetamol ($50mgkg^{-1}$) decreased neural discharge (spontaneous and mechanically-evoked) to 60-70% of pre-injection levels, the results of present study show that indomethacin ($10mgkg^{-1}$) produces much greater depressions (to 25-45% of pre-injection levels). An insufficient dose of aspirin or paracetamol used in the study by McQueen et al. (1991) cannot explain why these agents caused smaller depressions in neural discharge as compared with the present results using indomethacin, since McQueen et al. (1991) showed that lysine acetylsalicylate ($100mgkg^{-1}$) and paracetamol ($50mgkg^{-1}$) evidently caused maximal reductions in neural discharge as second doses of each agent failed to cause additional depression. One explanation that may account for the greater magnitude of depression in neural discharge seen with indomethacin is related to its anti-inflammatory ability; although indomethacin and aspirin have both analgesic and anti-inflammatory properties, it is indomethacin which has the greater anti-

inflammatory effect (Rang & Dale, 1991). Taking together the results of the present study and those of McQueen et al. (1991) it appears that the order of potency for the NSAIDs tested is indomethacin > lysine acetylsalicylate = paracetamol. A similar order of potency (indomethacin > lysine acetylsalicylate) has also been reported in afferents innervating inflamed cat knee joints (Heppelmann et al., 1986).

In the study by McQueen et al. (1991), reductions in mechanonociceptor responsiveness induced by lysine acetylsalicylate or paracetamol returned to pre-injection levels 60-80min post-injection of the drugs, whereas in the present experiments no recovery of resting or mechanically-evoked discharge was observed (maximal observation time 100min post-injection of indomethacin). These results, therefore, indicate that indomethacin has a longer duration of action than does lysine acetylsalicylate or paracetamol. This conclusion is also supported by the findings of Heppelmann et al. (1986) in afferent units innervating inflamed knee joints of cats, where there was no recovery of indomethacin-induced depressions in neural discharge within the observation period of 60 - 120min.

The present data showing that indomethacin is effective in reducing neural discharge from mechanonociceptors in arthritic joints is strong evidence for a peripheral action of the drug in or near the ankle joint. A peripheral site of action of other NSAIDs (lysine acetylsalicylate and paracetamol) has also been suggested in similar electrophysiological recordings from C- and Aδ-fibres innervating arthritic ankle joints (Guilbaud & Iggo, 1985; McQueen et al., 1991). However, electrophysiological

recordings from C-fibre neurons of the rat thalamus suggest that NSAIDs may also have central actions (Jurna & Brune, 1990).

Although NSAIDs were effective in reducing the sensitisation of mechano-nociceptors in inflamed joints in the present experiments and in those reported by Guilbaud & Iggo (1985) and McQueen et al. (1991), there remained residual neural discharge which was not affected by these agents. Since leukotrienes have been shown to sensitise mechanonociceptors (Martin et al., 1987), and since NSAIDs block cyclo-oxygenase activity (Vane, 1971; Collier and Schneider, 1972; Ferreira & Vane, 1974; Yamamoto et al., 1980; Vane & Botting, 1987) without having an affect on the enzyme 5-lipoxygenase [which leads to the formation of leukotrienes (Granström, 1983)], it is therefore possible that the NSAID-resistant component observed is mediated by leukotrienes. Further studies using dual inhibitors of cyclo-oxygenase and 5-lipoxygenase (e.g. 3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline, BW755C) would aid in elucidating the role of leukotrienes in this model of arthritis. The fact that NSAIDs could potentially divert arachidonate metabolism from the cyclo-oxygenase pathway to the lipoxygenase pathway, thereby increasing leukotriene production (Walker & Harvey, 1984) has therapeutic implications. Indeed a study (Rashad et al., 1989) of arthritic patients showed that arthritis progressed more rapidly in patients treated with a strong inhibitor of cyclo-oxygenase (indomethacin) than those treated with a weak inhibitor (azapropazone). Corticosteroid drugs have the ability to block the production of both prostaglandins and leukotrienes and therefore would be expected to show much greater anti-inflammatory properties than the NSAIDs. Indeed, as detailed in Section 6 of this thesis, the corticosteroid, dexamethasone,

shows much greater reductions in inflammation and hyperalgesia associated with adjuvant-arthritic joints as compared to indomethacin.

The results of the current investigation showed that indomethacin had no significant effect on the discharge (spontaneous and mechanically-evoked) from articular mechanonociceptors in normal joints. Similar electrophysiological recordings using other NSAIDs (lysine acetylsalicylate and paracetamol) have also shown that these agents do not alter neural discharge from joint capsule mechanonociceptors in normal joints (Guilbaud & Iggo, 1985; McQueen et al., 1991). The conclusion from electrophysiogical studies is that, unless inflammation is present NSAIDs will not affect neural discharge. In agreement with this conclusion is the observation that NSAIDs are only effective in experimental models which involve prior induction of inflammation (Randall & Selitto, 1957; Gilfoil et al., 1963; Winter, 1965; Pardo & Rodriguez, 1966; Vane & Botting, 1991). This is in contrast to opiate analgesics, where these drugs are analgesic both in inflamed and in normal tissues (Winter & Flataker, 1965; Vane & Botting, 1991).

### 4.4.2 Actions of indomethacin in adjuvant arthritis

In rats with established adjuvant-induced arthritis of the left ankle joint, treatment with indomethacin was found to reduce inflammation (Figures 4.8 & 4.9) and hyperalgesia (Figures 4.10 & 4.11). Such anti-inflammatory and analgesic actions induced by indomethacin have also been reported by other investigators using adjuvant-arthritic (Winter & Nuss, 1966; Kuzuna & Kawai, 1975; Wong & Gardocki,

1983; Elmadfa et al., 1987; Schmollack & Steup, 1988; Pettipher et al., 1989) or carrageenan, kaolin and urate-crystal (Rosenthale et al., 1972; Vinegar et al., 1976; Elling, 1987; Novo et al., 1992) models of joint inflammation in animals. In agreement with these animal studies, indomethacin has been demonstrated to be an effective analgesic and anti-inflammatory agent in patients with rheumatoid arthritis (Seideman & Eriksson, 1988; Seideman & Melander, 1988; Saul & Korlipara, 1991).

The finding that indomethacin-treated arthritic rats could sustain analgesia and reduced inflammation after withdrawal of the drug was interesting. Such an effect was also observed by Winter & Nuss (1966) after discontinuing indomethacin treatment in adjuvant-arthritic rats. Whether this could represent an effect of indomethacin on the underlying disease process rather than a mere anti-inflammatory action requires further investigation.

In the present experiments, the potency of indomethacin in this established model of arthritis was not determined in relation to other NSAIDs such as aspirin. Nevertheless, it has been determined using various parameters of inflammation and hyperalgesia in an established model of adjuvant-induced arthritis in rats, that the order of potency is indomethacin > aspirin (Winter & Nuss, 1966; Kuzuna & Kawai, 1975; Elmadfa et al., 1987). This order of potency agrees with that observed in the present electrophysiological studies (see Section 4.3.1).

### 4.4.3 Mechanism(s) of action of indomethacin

The precise mechanism(s) of action of indomethacin in both the present electrophysiological and behavioural studies requires investigation. NSAIDs have been shown to inhibit prostanoid synthesis (Vane, 1971; Ferreira & Vane, 1974; Moncada et al., 1975; Fitzpatrick & Wynalda, 1976; Sturge et al., 1978; Van der Ouderas et al., 1980; Vane & Botting, 1991) and are believed to cause reductions in tissue prostanoid content resulting in analgesia (Collier et al., 1972; Ferreira & Vane, 1974; Moncada et al., 1975; Vane & Botting, 1991). Electrophysiological studies using adjuvant-arthritic rat ankle joints have shown that the stable and selective IP receptor agonist, cicaprost (Skubulla et al., 1986), restored neural discharge which had been reduced by lysine acetylsalicylate (McQueen et al., 1991). This result suggests that IP receptors are involved in the sensitisation of mechanonociceptors in this model of arthritis. Reversal by $PGE_2$ of indomethacin-induced decreases in neural discharge from inflamed (kaolin-carrageenan induced) cat knee joints indicates that EP receptors may also be involved in the sensitisation process (Heppelmann et al., 1986). However, no reversal was obtained with $PGE_2$ in electrophysiological studies using adjuvant-arthritic rat joints (McQueen et al., 1991), indicating that sensitisation of mechanonociceptors is dependent on the inflammatory stimulus and / or species used.

As discussed by Lands (1981; 1985) inhibition of cyclo-oxygenase by NSAIDs can occur by different mechanisms such as irreversible inactivation, reversible competitive inhibition and reversible non-competitive inhibition. Aspirin acts through an

irreversible inactivation of the enzyme - it acetylates the α-amino group of the terminal serine to form a covalent bond. Thus, the effects of aspirin continue after the drug itself has apparently been cleared from the tissue. A similar effect as aspirin has been observed with indomethacin on isolated cyclo-oxyygenase (Lands, 1985). However, on whole cells the action of indomethacin on cyclo-oxygenase appears to be reversible (Lands, 1985).

Although it is generally accepted that the mechanism of action of NSAIDs is via inhibition of cyclo-oxygenase several other mechanisms (at equivalent doses) may also be involved. For example, NSAIDs can inhibit enzymes other than cyclo-oxygenase (Kuehl & Egan, 1980; Brune, 1983). Other mechanisms of action of NSAIDs include their ability to act directly on neuronal membranes (Neto & Narahashi, 1976), to counteract the effects of excitatory substances (Guzman et al., 1964; Lim et al., 1970), and to reduce intracellular free $Ca^{2+}$ (Northover, 1971;1977).

## 4.5 SUMMARY

In summary, the present electrophysiological results provide evidence that indomethacin has an action in the periphery to reduce the elevated spontaneous discharge, and the enhanced responsiveness to mechanical stimuli, of articular mechanonociceptors in chronically arthritic (Freunds adjuvant-induced) rat ankle joints. In line with these neuropharmacological studies, complementary behavioural studies showed that indomethacin reduced the hyperalgesia, swelling and

inflammation associated with an established monoarthritis of the rat ankle joint induced by Freunds adjuvant.

## *SECTION 5*

# *PURINE RECEPTORS AND ARTICULAR MECHANONOCICEPTOR DISCHARGE FROM NORMAL AND ARTHRITIC RAT ANKLE JOINTS.*

## 5.1 INTRODUCTION

Observations by Bleehen & Keele (1977) have shown that pain results from the application of adenosine to a cantharadin-induced blister base of the human forearm. Pain can also be evoked in humans when adenosine is injected into the coronary (Crea et al., 1990; Lagerqvist et al., 1990a), brachial (Sylvén et al., 1988b) and iliac (Lagerqvist et al., 1990a) arteries, or when it is injected intravenously (Conradsson et al., 1987; Sylvén et al., 1988a; Crea et al., 1990; Lagerqvist et al., 1990b) or into the abdominal aorta (Lagerqvist et al., 1990a).

Purinoceptors are classified into the $P_1$ (adenosine receptors) and $P_2$ (ATP receptors) types (Burnstock & Kennedy, 1985; Burnstock, 1990; Hoyle & Burnstock, 1991; Stiles, 1991). Adenosine receptors are further subclassified into the $A_1$, $A_2$ and $A_3$ subtypes with evidence emerging for further divisions of these subtypes (Hamprecht & Calker, 1985; Linden, 1991; Collis & Hourani, 1993). Behavioural studies in rats, have shown that intra-dermal injections of adenosine causes direct cutaneous mechanical hyperalgesia (Taiwo & Levine, 1990). This hyperalgesia was mimicked by $A_2$ receptor agonists and reversed by $A_2$ receptor antagonists , whereas $A_1$ selective adenosine analogues were without effect. Various electrophysiological studies in animals have also demonstrated an excitatory role for adenosine [e.g. there is activation of carotid body (McQueen & Ribeiro, 1981; Moteiro & Ribeiro, 1987), vagal pulmonary (Cherniack et al., 1987; Runold et al., 1987) and renal pelvis (Katholi et al., 1985) afferents].

Paradoxically, it has been reported that adenosine or adenosine agonists ($A_1$ and/or $A_2$ selective) have antinociceptive or anti-inflammatory actions in various animal models, including carrageenan-induced pleurisy (Schrier et al., 1990; Lesch et al., 1991) or paw inflammation (Firestein et al., 1993) and tail flick (Holmgren et al, 1983) or hot plate (Holmgren et al., 1986) tests in rats, and acetylcholine-induced writhing (Herrick-Davis et al., 1989) or substance P or N-Methyl-D-aspartate-induced nociceptive behaviour (Delander & Wahl, 1988) in mice.

The main aim of the present investigation was to determine the effects of adenosine and agonists and antagonists selective for adenosine receptors on the discharge (spontaneous and mechanically-evoked) from articular mechanonociceptors in normal rat ankle joints as well as in ankle joints with localised adjuvant-induced arthritis. Preliminary studies were also performed to determine the effects of the $P_2$ receptor agonists, ATP and $\alpha,\beta$-methylene-ATP, on mechanonociceptor responsiveness in both normal and arthritic rat ankle joints.

## 5.2 MATERIALS & METHODS

### 5.2.1 Electrophysiological studies

The in-vivo preparation, neural recording, off-line analysis and statistical analysis are described in detail in Section 2. In brief, male Wistar rats (normal and adjuvant-arthritic) were anaesthetised with urethane and cannulations performed of the trachea, right carotid artery (blood pressure monitoring) and right femoral artery (retrograde cannulation for close intra-arterial bolus injections of drugs into the left limb). The medial aspect of the left ankle joint was exposed and nerve fibres were isolated from the PACR nerve. C-fibre afferent discharge (spontaneous and mechanically-evoked) from articular mechanonociceptors was recorded extracellularly, using bipolar platinum-iridium electrodes.

**Protocol**

The protocol in these experiments involved an attempt to construct log dose-response curves to the non-selective adenosine receptor agonist, 5`-N-ethylcarboxamidoadenosine (NECA, 0.01 - 100μg i.a.), in both normal and arthritic rats. In some of the experiments, the selective $A_1$ receptor agonist, $N^6$-cyclopentyladenosine (CPA), and the agonist, metrifudil ($A_2 > A_1$), were injected (10μg, i.a.) either before, or more typically, after injections of NECA (0.01 - 100μg). The effects of the non-selective adenosine receptor antagonists, theophylline and 8-phenyltheophylline (8-PT, 100μgkg$^{-1}$ i.a.), were also determined. In a series of preliminary experiments, the effects of the non-selective $P_2$ agonist, ATP (100μg,

i.a.), and the $P_{2x}$ selective agonist, α,β-methylene-ATP (100μg, i.a.), were determined in both normal and arthritic rats.

**Data analysis**

In this series of experiments, if the test agent evoked an obvious change in spontaneous discharge then this was assessed by determining the number of impulses the test agent induced above, or below, the control level of discharge (see Section 2.3.5.2 for further details of the formula used) with the delay and duration of the response also being determined. If the test agent showed no clear response, then the largest number of impulses both above and below basal discharge was determined by using an arbitrary time period of 60s over 0 - 15min post-injection of drug. The control period was defined as the 60s period immediately prior to the addition of the test substance. A significant change in spontaneous discharge was defined as a change over basal discharge of more than 5 impulses for normal joints and over 6 impulses for arthritic joints. These values of 5 and 6 impulses were derived from the results of saline injections in normal (3 ± 2 and 4 ± 1 impulses above and below basal discharge, respectively, 7 units) and arthritic (4 ± 2 and 5 ± 1 impulses above and below basal discharge, respectively, 11 units) joints. Effects of the test substances on the responsiveness of the standard mechanical stimulus were assessed as the peak number of impulses above, or below, the pre-injection evoked discharge. A significant change in mechanically-evoked discharge was defined as a change of more than 5 impulses over the basal mechanically-evoked discharge for both normal and arthritic joints. This value of 5 impulses, was derived from the results of saline injections in normal (4

± 1 and 4 ± 1 impulses above and below basal discharge, respectively, 6 units) and arthritic (4 ± 1 and 3 ± 1 impulses above and below basal discharge, respectively, 9 units) joints.

## 5.3 RESULTS

### 5.3.1 *In-vivo* electrophysiology in normal and adjuvant-arthritic rat ankle joints

In this series of experiments the effects of adenosine agonists and antagonists were examined in 11 single units (10 experiments) from normal joints and 16 single units (14 experiments) from adjuvant-arthritic (mean 26 ± 9 days post-adjuvant) joints. Although conduction velocities of the action potential spikes recorded in this series of experiments were not determined, it is likely that they were in the C- or Aδ-fibre range since a) the action potential spike shape characteristics and duration of all the articular afferent units recorded were similar to those of C- or A-δ fibres (see Section 3) and b) all the units examined showed chemosensitivity to the C-fibre excitant, capsaicin (1 - 3μg, i.a.). Before the addition of any drugs, all units showed resting (spontaneous) discharge, although this was significantly greater in units from arthritic joints (3.6 ± 1.3 i.p.s.; range: 1.4 - 5.5) than in those from normal joints (0.8 ± 0.2 i.p.s.; range: 0.3 - 1.5)[$P<0.05$, Mann-Whitney U-test].

#### 5.3.1.1 Effects of adenosine receptor agonists and antagonists on mechanonociceptor discharges from normal joints

In comparison with saline injections, none of the units recorded from normal joints showed any significant change in either spontaneous or mechanically-evoked discharge after close arterial injections of the non-selective adenosine agonist, NECA (0.01 - 30μg; Tables 5.1 & 5.2), the selective $A_1$ receptor agonist, CPA (10μg; Tables 5.3 & 5.4), and the $A_2 > A_1$ receptor agonist, metrifudil (10μg;

**Table 5.1 Lack of effect of NECA on spontaneous discharge from articular mechanonociceptors in normal rat ankle joints.**

| | | *units examined (n)* | *impulses above basal discharge* | *† P* | *impulses below basal discharge* | *† P* |
|---|---|---|---|---|---|---|
| saline | | 7 (6) | 3 ± 2 | - | 4 ± 1 | - |
| NECA | 0.01μg | 5 (5) | 3 ± 1 | NS | 3 ± 1 | NS |
| NECA | 0.1μg | 6 (6) | 3 ± 1 | NS | 4 ± 1 | NS |
| NECA | 1μg | 6 (6) | 4 ± 1 | NS | 4 ± 1 | NS |
| NECA | 10μg | 7 (6) | 3 ± 2 | NS | 4 ± 1 | NS |
| NECA | 30μg | 6 (6) | 4 ± 1 | NS | 3 ± 1 | NS |

*†* Statistical comparisons (Mann Whitney U-test) between saline and 5`-N-ethylcarboxamidoadenosine (NECA). NS = P>0.05. The pre-injection discharge, before the injection of any drugs, was 0.9 ± 0.2 i.p.s.

**Table 5.2 Lack of effect of NECA on mechanically-evoked discharge from articular mechanonociceptors in normal rat ankle joints.**

| | | *units examined (n)* | *impulses above evoked basal discharge* | *† P* | *impulses below evoked basal discharge* | *† P* |
|---|---|---|---|---|---|---|
| saline | | 4 (4) | 3 ± 1 | - | 3 ± 1 | - |
| NECA | 0.01μg | 4 (4) | 1 ± 2 | NS | 3 ± 2 | NS |
| NECA | 0.1μg | 4 (4) | -1 ± 2 | NS | 3 ± 2 | NS |
| NECA | 1μg | 4 (4) | 2 ± 3 | NS | 2 ± 2 | NS |
| NECA | 10μg | 4 (4) | 2 ± 2 | NS | 4 ± 1 | NS |
| NECA | 30μg | 3 (3) | -1 ± 2 | NS | 4 ± 1 | NS |

*†* Statistical comparisons (Mann Whitney U-test) between saline and 5`-N-ethylcarboxamidoadenosine (NECA). NS = P>0.05. The pre-injection mechanically-evoked discharge, before the injection of any drugs, was 48 ± 8 impulses.

**Table 5.3 Lack of effect of CPA and metrifudil on spontaneous discharge from articular mechanonociceptors in normal rat ankle joints.**

| | *units examined (n)* | *impulses above basal discharge* | *† P* | *impulses below basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 4 (4) | 3 ± 1 | - | 4 ± 1 | - |
| CPA 10μg | 3 (3) | 4 ± 1 | NS | 3 ± 2 | NS |
| metrifudil 10μg | 3 (3) | 3 ± 2 | NS | 1 ± 2 | NS |

† Statistical comparisons (Mann-Whitney U-test) between saline and the test adenosine agonist. NS = P>0.05. CPA: $N^6$-cyclopentyladenosine. The pre-injection discharge, was 1.1 ± 0.4 i.p.s pre-saline, 0.7 ± 0.2 i.p.s. pre-CPA and 1.0 ± 0.4 i.p.s. pre-metrifudil.

**Table 5.4 Lack of effect of CPA and metrifudil on mechanically-evoked discharge from articular mechanonociceptors in normal rat ankle joints.**

| | *units examined (n)* | *impulses above evoked basal discharge* | *† P* | *impulses below evoked basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 3 (3) | 3 ± 1 | - | 4 ± 1 | - |
| CPA 10μg | 2 (2) | 4 ± 1 | NS | 3 ± 2 | NS |
| metrifudil 10μg | 2 (2) | 2 ± 1 | NS | 5 ± 0 | NS |

† Statistical comparisons (Mann-Whitney U-test) between saline and the test adenosine agonist. NS = P>0.05. CPA: $N^6$-cyclopentyladenosine. The pre-injection mechanically-evoked discharge, was 48 ± 17 impulses pre-saline, 36 ± 19 impulses pre-CPA and 43 ± 21 impulses pre-metrifudil.

Tables 5.3 & 5.4). The non-selective adenosine receptor antagonists, theophylline and 8-PT (100μgkg$^{-1}$, i.a.), also had no significant effect on either spontaneous (Table 5.5) or mechanically- evoked discharge (Table 5.6).

#### 5.3.1.2 Effects of adenosine receptor agonists and antagonists on mechanonociceptor discharges from arthritic joints

In all the units recorded from arthritic joints, there was no significant effect (in comparison with saline injections) on either spontaneous discharge or on the responsiveness to the standard mechanical stimulus after close intra-arterial injections of NECA (0.01 - 100μg; Tables 5.7 & 5.8), CPA (10μg; Tables 5.9 & 5.10) and metrifudil (10μg; Tables 5.9 & 5.10). The non-selective adenosine receptor antagonists, theophylline and 8-PT (100μgkg$^{-1}$, i.a.), had no significant effect on either spontaneous discharge (Table 5.11) or on the responsiveness to the standard mechanical stimulus (Table 5.12).

#### 5.3.1.3 Effects of ATP and α,β-methylene-ATP on mechanonociceptor discharges in normal and arthritic rat ankle joints

In preliminary studies, a high dose (100μg, i.a.) of either the non-selective $P_2$ receptor agonist, ATP, or the selective $P_{2x}$ receptor agonist, α,β-methylene-ATP, failed to cause any significant change in spontaneous or mechanically-evoked discharge in any of the units recorded from either normal (Tables 5.13 & 5.14) or arthritic (Tables 5.15 & 5.16) joints.

**Table 5.5 Lack of effect of theophylline and 8-PT on spontaneous discharge from articular mechanonociceptors in normal rat ankle joints.**

| | *units examined (n)* | *impulses above basal discharge* | *† P* | *impulses below basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 5 (5) | 4 ± 1 | - | 3 ± 1 | - |
| theophylline 100μgkg$^{-1}$ | 5 (5) | 2 ± 1 | NS | 4 ± 1 | NS |
| 8-PT 100μgkg$^{-1}$ | 3 (3) | 3 ± 1 | NS | 4 ± 1 | NS |

*†* Statistical comparisons (Mann-Whitney U-test) between saline and the test adenosine antagonist. NS = P>0.05. 8-PT: 8-phenyltheophylline. The pre-injection discharge, was 1.2 ± 0.3 i.p.s pre-saline, 1.2 ± 0.2 i.p.s. pre-theophylline and 1.2 ± 0.5 i.p.s. pre-8-PT.

**Table 5.6 Lack of effect of theophylline and 8-PT on mechanically-evoked discharge from articular mechanonociceptors in normal rat ankle joints.**

| | *units examined (n)* | *impulses above evoked basal discharge* | *† P* | *impulses below evoked basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 3 (3) | 3 ± 1 | - | 4 ± 1 | - |
| theophylline 100μgkg$^{-1}$ | 3 (3) | 0 ± 3 | NS | 3 ± 2 | NS |
| 8-PT 100μgkg$^{-1}$ | 3 (3) | 4 ± 1 | NS | 3 ± 2 | NS |

*†* Statistical comparisons (Mann-Whitney U-test) between saline and the test adenosine antagonist. NS = P>0.05. 8-PT: 8-phenyltheophylline. The pre-injection mechanically-evoked discharge, was 50 ± 13 impulses pre-saline, 36 ± 14 impulses pre-theophylline and 43 ± 15 impulses pre-8-PT.

**Table 5.7 Lack of effect of NECA on spontaneous discharge from articular mechanonociceptors in arthritic rat ankle joints.**

| | | *units examined (n)* | *impulses above basal discharge* | *† P* | *impulses below basal discharge* | *† P* |
|---|---|---|---|---|---|---|
| saline | | 11 (9) | 4 ± 2 | - | 5 ± 1 | - |
| NECA | 0.01μg | 5 (3) | 2 ± 2 | NS | 3 ± 1 | NS |
| NECA | 0.1μg | 9 (7) | 4 ± 1 | NS | 5 ± 1 | NS |
| NECA | 1μg | 9 (7) | 4 ± 1 | NS | 4 ± 2 | NS |
| NECA | 10μg | 11 (9) | 4 ± 2 | NS | 4 ± 2 | NS |
| NECA | 30μg | 6 (4) | 4 ± 2 | NS | 5 ± 1 | NS |
| NECA | 100μg | 5 (3) | 5 ± 1 | NS | 4 ± 2 | NS |

† Statistical comparisons (Mann Whitney U-test) between saline and 5`-N-ethylcarboxamidoadenosine (NECA). NS = P>0.05. The pre-injection discharge, before the injection of any drugs, was 3.7 ± 0.5 i.p.s.

**Table 5.8 Lack of effect of NECA on mechanically-evoked discharge from articular mechanonociceptors in arthritic rat ankle joints.**

| | | *units examined (n)* | *impulses above evoked basal discharge* | *† P* | *impulses below evoked basal discharge* | *† P* |
|---|---|---|---|---|---|---|
| saline | | 7 (6) | 2 ± 1 | - | 3 ± 1 | - |
| NECA | 0.01μg | 3 (2) | 1 ± 1 | NS | 4 ± 1 | NS |
| NECA | 0.1μg | 5 (4) | 1 ± 3 | NS | 4 ± 1 | NS |
| NECA | 1μg | 5 (4) | 2 ± 3 | NS | 2 ± 1 | NS |
| NECA | 10μg | 7 (6) | 1 ± 2 | NS | 2 ± 1 | NS |
| NECA | 30μg | 4 (3) | 2 ± 3 | NS | 3 ± 1 | NS |
| NECA | 100μg | 4 (3) | 3 ± 1 | NS | 4 ± 1 | NS |

† Statistical comparisons (Mann Whitney U-test) between saline and 5`-N-ethylcarboxamidoadenosine (NECA). NS = P>0.05. The pre-injection mechanically-evoked discharge, before the injection of any drugs, was 55 ± 10 impulses.

**Table 5.9 Lack of effect of CPA and metrifudil on spontaneous discharge from articular mechanonociceptors in arthritic rat ankle joints.**

| | *units examined (n)* | *impulses above basal discharge* | *† P* | *impulses below basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 6 (5) | 5 ± 1 | - | 4 ± 1 | - |
| CPA 10μg | 6 (5) | 4 ± 2 | NS | 5 ± 1 | NS |
| metrifudil 10μg | 5 (4) | 4 ± 2 | NS | 4 ± 2 | NS |

† Statistical comparisons (Mann-Whitney U-test) between saline and the test adenosine agonist. NS = P>0.05. CPA: $N^6$-cyclopentyladenosine. The pre-injection discharge, was 2.2 ± 0.4 i.p.s pre-saline, 2.2 ± 0.1 i.p.s. pre-CPA and 2.2 ± 0.3 i.p.s. pre-metrifudil.

**Table 5.10 Lack of effect of CPA and metrifudil on mechanically-evoked discharge from articular mechanonociceptors in arthritic rat ankle joints.**

| | *units examined (n)* | *impulses above evoked basal discharge* | *† P* | *impulses below evoked basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 6 (5) | 3 ± 1 | - | 3 ± 1 | - |
| CPA 10μg | 6 (5) | 4 ± 1 | NS | 3 ± 1 | NS |
| metrifudil 10μg | 5 (4) | 4 ± 1 | NS | 2 ± 1 | NS |

† Statistical comparisons (Mann-Whitney U-test) between saline and the test adenosine agonist. NS = P>0.05. CPA: $N^6$-cyclopentyladenosine. The pre-injection mechanically-evoked discharge, was 37 ± 9 impulses pre-saline, 26 ± 6 impulses pre-CPA and 30 ± 3 impulses pre-metrifudil.

**Table 5.11 Lack of effect of theophylline and 8-PT on spontaneous discharge from articular mechanonociceptors in arthritic rat ankle joints.**

| | *units examined (n)* | *impulses above basal discharge* | *† P* | *impulses below basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 11 (10) | 4 ± 1 | - | 3 ± 1 | - |
| theophylline 100μgkg$^{-1}$ | 6 (5) | 3 ± 2 | NS | 4 ± 2 | NS |
| 8-PT 100μgkg$^{-1}$ | 5 (5) | 4 ± 2 | NS | 4 ± 2 | NS |

*†* Statistical comparisons (Mann-Whitney U-test) between saline and the test adenosine antagonist. NS = P>0.05. 8-PT: 8-phenyltheophylline. The pre-injection discharge, was 2.8 ± 0.3 i.p.s pre-saline, 3.1 ± 0.4 i.p.s. pre-theophylline and 2.4 ± 0.5 i.p.s. pre-8-PT.

**Table 5.12 Lack of effect of theophylline and 8-PT on mechanically-evoked discharge from articular mechanonociceptors in arthritic rat ankle joints.**

| | *units examined (n)* | *impulses above evoked basal discharge* | *† P* | *impulses below evoked basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 11 (9) | 3 ± 1 | - | 3 ± 1 | - |
| theophylline 100μgkg$^{-1}$ | 5 (4) | 4 ± 1 | NS | 1 ± 2 | NS |
| 8-PT 100μgkg$^{-1}$ | 6 (5) | 3 ± 1 | NS | 2 ± 1 | NS |

*†* Statistical comparisons (Mann-Whitney U-test) between saline and the test adenosine antagonist. NS = P>0.05. 8-PT: 8-phenyltheophylline. The pre-injection mechanically-evoked discharge, was 39 ± 4 impulses pre-saline, 44 ± 12 impulses pre-theophylline and 30 ± 6 impulses pre-8-PT.

**Table 5.13 Lack of effect of ATP & α,β-methylene-ATP on spontaneous discharge from articular mechanonociceptors in normal rat ankle joints.**

| | *units studied (n)* | *impulses above basal discharge* | *† P* | *impulses below basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 5 (5) | 3 ± 1 | - | 4 ± 1 | - |
| ATP 100μg | 1 (1) | 3 ± 1 | NS | 3 ± 1 | NS |
| α,β-methylene-ATP 100μg | 5 (5) | 4 ± 1 | NS | 2 ± 1 | NS |

† Statistical comparisons (Mann-Whitney U-test) between saline and the test $P_2$ agonist.
NS = P>0.05. The pre-injection discharge, was 0.6 ± 0.3 i.p.s pre-saline, 0.8 ± 0.2 i.p.s. pre-ATP and 0.5 ± 0.2 i.p.s. pre-α,β-methylene-ATP.

**Table 5.14 Lack of effect of ATP & α,β-methylene-ATP on mechanically-evoked discharge from articular mechanonociceptors in normal rat ankle joints.**

| | *units studied (n)* | *impulses above evoked basal discharge* | *† P* | *impulses below evoked basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 4 (4) | 3 ± 1 | - | 4 ± 1 | - |
| ATP 100μg | 1 (1) | 2 ± 1 | NS | 4 ± 1 | NS |
| α,β-methylene-ATP 100μg | 4 (4) | 2 ± 2 | NS | 3 ± 1 | NS |

† Statistical comparisons (Mann-Whitney U-test) between saline and the test $P_2$ agonist.
NS = P>0.05. The pre-injection mechanically-evoked discharge, was 25 ± 6 impulses pre-saline, 29 ± 8 impulses pre-ATP and 17 ± 7 impulses pre-α,β-methylene-ATP.

**Table 5.15 Lack of effect of ATP & α,β-methylene-ATP on spontaneous discharge from articular mechanonociceptors in arthritic rat ankle joints.**

| | *units studied (n)* | *impulses above basal discharge* | *† P* | *impulses below basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 7 (6) | 2 ± 1 | - | 3 ± 1 | - |
| ATP 100μg | 2 (2) | 3 ± 1 | NS | 4 ± 1 | NS |
| α,β-methylene-ATP 100μg | 7 (6) | 4 ± 1 | NS | 4 ± 2 | NS |

† Statistical comparisons (Mann-Whitney U-test) between saline and the test $P_2$ agonist.
NS = P>0.05. The pre-injection discharge, was 2.4 ± 0.6 i.p.s pre-saline, 2.6 ± 0.9 pre-ATP and 2.1 ± 1.1 i.p.s. pre-α,β-methylene-ATP.

**Table 5.16 Lack of effect of ATP & α,β-methylene-ATP on mechanically-evoked discharge from articular mechanonociceptors in arthritic rat ankle joints.**

| | *units studied (n)* | *impulses above evoked basal discharge* | *† P* | *impulses below evoked basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 4 (3) | 4 ± 1 | - | 3 ± 2 | - |
| ATP 100μg | 2 (2) | 3 ± 1 | NS | 4 ± 1 | NS |
| α,β-methylene-ATP 100μg | 4 (3) | 4 ± 1 | NS | 2 ± 1 | NS |

† Statistical comparisons (Mann-Whitney U-test) between saline and the test $P_2$ agonist.
NS = P>0.05. The pre-injection mechanically-evoked discharge, was 55 ± 25 impulses pre-saline, 48 ± 22 impulses pre-ATP and 42 ± 21 impulses pre-α,β-methylene-ATP.

### 5.3.2 Blood pressure studies

Intra-arterial injection of either NECA (0.01 - 100μg) or metrifudil (10μg) caused a fall in systemic blood pressure, whereas CPA (10μg) caused an increase. These effects to adenosine receptor agonists were antagonised by theophylline or 8-PT ($100\mu gkg^{-1}$, i.a.)(see Figure 5.1 for typical responses). The $P_2$ receptor agonists, ATP and α,β-methylene-ATP (100μg, i.a.), caused a decrease and increase, respectively, in systemic blood pressure (see Figure 5.2 for typical responses).

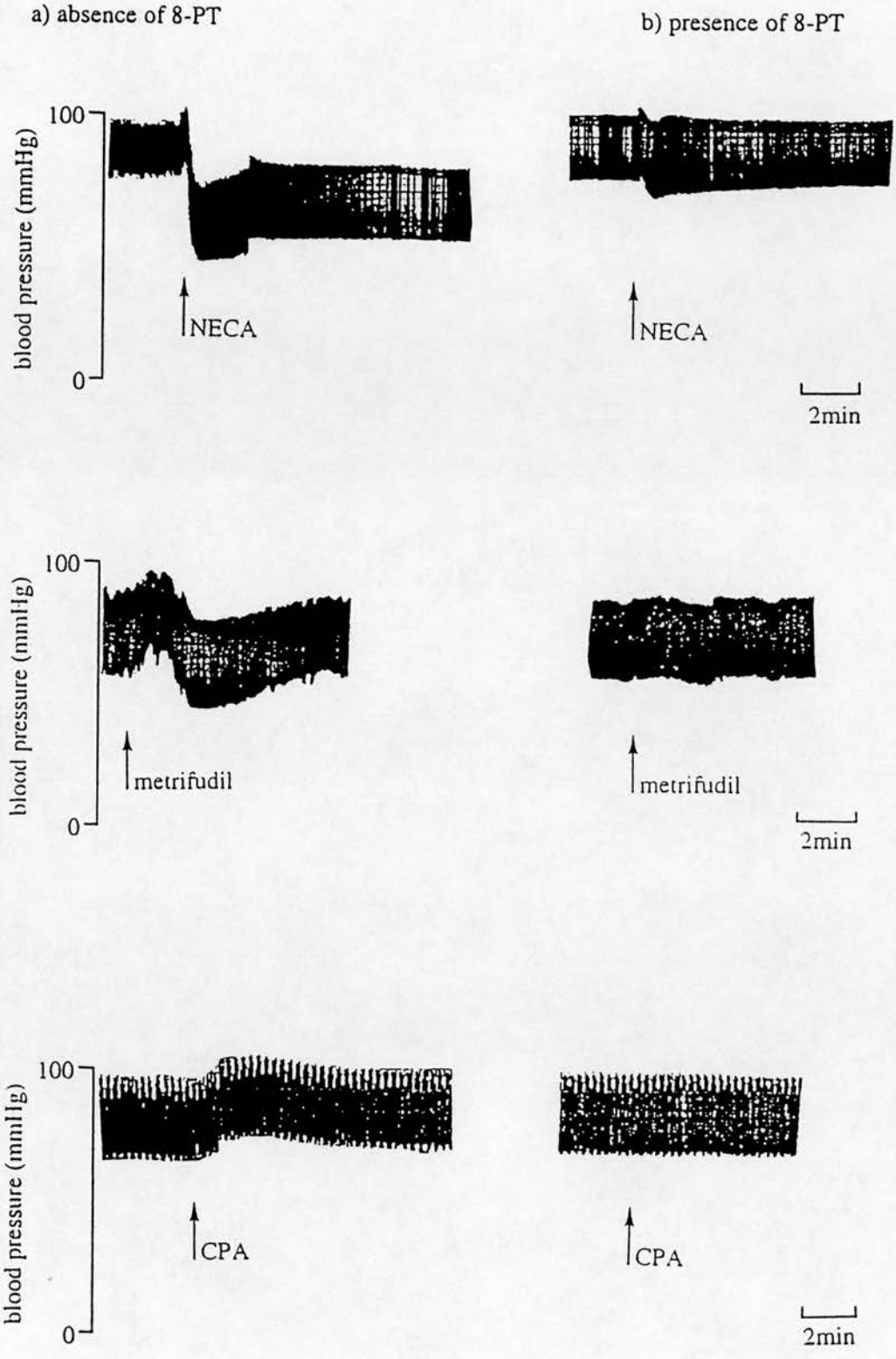
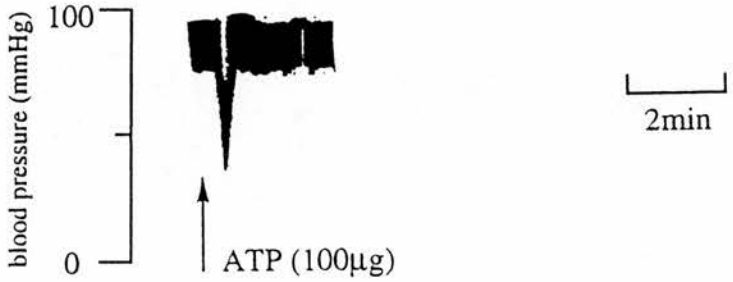



Figure 5.1 Chart recording traces showing typical responses to intra-arterial injection of NECA (10μg), metrifudil (10μg) and CPA (10μg) on systemic blood pressure (a) before and (b) after 8-PT ($100\mu gkg^{-1}$, i.a.). The traces in the above figures were all obtained from arthritic rats (normal rats also showed similar responses).

a)




b)

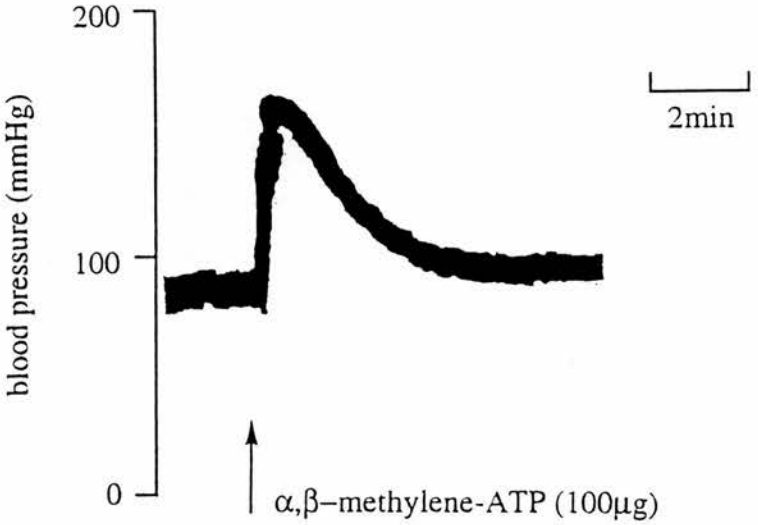



Figure 5.2 Chart recording traces showing the typical changes in systemic blood pressure associated with intra-arterial injection (100μg) of ATP (a) and α,β,-methylene-ATP (b). The traces in the above figures were from arthritic rats (normal rats also showed similar responses).

## 5.4 DISCUSSION

### 5.4.1 Adenosine receptor agonists and antagonists on mechanonociceptor discharge from normal and adjuvant-arthritic rat ankle joints

The results of the present investigation have shown that none of the adenosine receptor agonists (non-selective: NECA; $A_1$-selective: CPA; $A_2 > A_1$ selective: metrifudil) had any significant effect on spontaneous or mechanically-evoked discharge from mechanonociceptors in either normal or chronically inflamed (adjuvant-arthritic) rat ankle joints. These results are in agreement with those of Klement & Arndt (1992), who found that adenosine injected into the human finger vein failed to evoke pain from venous and paravascular nociceptors, nor did it alter the intensity of electrically-evoked pain. Since non-selective adenosine receptor antagonists (theophylline and 8-PT) had no effect on either the enhanced spontaneous discharge or the sensitisation of mechanonociceptors to mechanical stimuli seen in chronically arthritic ankle joints, these results suggest that, in this model of chronic inflammation, adenosine and adenosine receptors are unlikely to play a significant role in the excitation or sensitisation of mechanonociceptors.

It is possible that a change in neural discharge was not observed in the current experiments because adenosine analogues, even when injected at high concentrations, failed to reach effective concentrations at the nociceptor terminals in the ankle joint because of, for example, rapid degradation of the analogues. However, this is unlikely since stable adenosine agonists such as NECA produced prolonged changes in blood pressure (Figure 5.1). Moreover, since the blood pressure effects to the adenosine

receptor agonists were antagonised by theophylline or 8-PT (Figure 5.1) this shows that these antagonists were bioactive in the concentrations used in the experiments. The possibility that articular afferent units studied in this series of experiments were incapable of showing alterations in neural discharge is unlikely, since chemosensitivity of all the units recorded was confirmed by responsiveness to the C-fibre excitant, capsaicin.

In contrast to the findings of the present study, it has been shown that adenosine can evoke pain in humans when applied to the blister base (Bleehen & Keele, 1977) or when injected intra-arterially (Sylvén et al., 1988b; Crea et al., 1990; Lagerqvist et al., 1990a) or intravenously (Conradsson et al., 1987; Sylvén et al., 1988a; Crea et al., 1990; Lagerqvist et al., 1990b). In further contrast with the results of the current investigation, it has been reported that intra-dermal injection of adenosine causes direct cutaneous mechanical hyperalgesia in the rat, an effect mediated by $A_2$ receptors (Taiwo & Levine, 1990). A central mode of action might explain why adenosine had effects in these studies but lacked action in the present experiments, which examined the effects of adenosine only on peripheral tissue. However, it appears that when $A_1$ and/or $A_2$ selective agonists are injected intracerebroventrically (Herrick-Davis et al., 1989) or intrathecally (Holmgren et al., 1986; Sawynok et al., 1986) antinociception (tail flick, hot plate or acetylcholine-induced writhing tests), rather than nociception, results. It would be of interest to perform behavioural studies using the Freunds adjuvant-induced mono-arthritic rat model (see Section 3) so as to determine what effects, if any, adenosine agonists and antagonists would have in this

model of chronic inflammation, and then to relate these results to those of the present neuropharmacological study.

It has been shown that, although $PGE_1$ or $PGE_2$ cause little or no increase in afferent discharge, they can cause potentiation of the excitatory response to bradykinin (Chahl & Iggo, 1977; Birrell et al., 1993). Thus, although in the present experiments adenosine analogues caused no change in neural discharge from articular mechanonociceptors, it may be the case that adenosine could affect afferent discharge to other inflammatory agents such as bradykinin, prostanoids or 5-HT. Further neuropharmacological studies will be required to test this hypothesis.

### 5.4.2 Role of $P_2$ receptors on mechanonociceptor discharges from normal and adjuvant-arthritic rats

Purinoceptors at which ATP acts are classified as $P_2$ receptors. These $P_2$ receptors have been classified into two subtypes, $P_{2x}$ and $P_{2y}$ (O'Conner et al., 1991; O'Conner, 1992; Illes & Nörenberg, 1993). It has been shown that application of the non-selective $P_2$ receptor agonist, ATP, to the human blister base produces pain (Bleehen & Keele, 1977). In various sensory neurons, it has been demonstrated that $P_2$ receptors (in particular $P_{2x}$ subtypes) are activated by ATP and ATP analogues (Krishtal et al., 1988; Burnstock, 1990; Illes & Nörenberg, 1993). ATP, and the selective $P_{2x}$ receptor agonist, $\alpha,\beta$-methylene-ATP, have also been reported to cause depolarisation of the rat isolated vagus (Trezise et al., 1993). In this study, depolarisations were blocked by the non-selective $P_2$ receptor antagonist, suramin. In

contrast to these reports in the literature, preliminary findings of the present investigation have shown that $P_2$ receptors are unlikely to have any significant role in modulating articular afferent discharge because close intra-arterial injection of the non-selective $P_2$ receptor agonist ATP, or of a selective $P_{2x}$ receptor agonist ($\alpha,\beta$-methylene-ATP) failed to alter articular afferent discharge from mechanonociceptors in either normal or arthritic rat ankle joints. Although ATP and $\alpha,\beta$-methylene-ATP had no effect on neural discharge, they were bioactive since they caused a decrease and an increase, respectively, in systemic blood pressure (Figure 5.2). Further experiments examining the role of $P_2$ receptor antagonists such as suramin or arylazidoamino propionyl-ATP ($ANAPP_3$) would be of interest, in order to determine whether ATP could be involved in the tonic sensitisation of mechanonociceptors in chronically inflamed joints.

## 5.5 SUMMARY

In summary, the results of the present neuropharmacological investigation provide strong evidence that neither adenosine $A_1$ or $A_2$ receptors (subtypes of the $P_1$ receptor) affect neural discharge from mechanonociceptors in either normal or arthritic rat ankle joints. Preliminary studies indicate that purine $P_2$ receptors are also unlikely to be involved in altering articular mechanonociceptor discharge in either normal or chronically inflamed joints.

## *SECTION 6*

# *ROLE OF β-ADRENOCEPTORS IN NORMAL AND ADJUVANT-ARTHRITIC RAT ANKLE JOINTS: ELECTROPHYSIOLOGICAL AND BEHAVIOURAL STUDIES.*

## 6.1 INTRODUCTION

In humans, adrenoceptors have been implicated in chronic inflammatory disease states such as reflex sympathetic dystrophy (Schott, 1986; Schwartzman & McLellan, 1987) and rheumatoid arthritis (Kaplan et al., 1980; Levine et al., 1986a; Hannington-Kiff, 1990). In these disorders a reduction in pain and inflammation can be achieved by the use of adrenergic neurone blocking drugs, such as guanethidine (Loh & Nathan, 1978; Bonica, 1979; Levine et al., 1986a Hannington-Kiff, 1990).

A contribution of adrenoceptors, particularly $\beta_2$-adrenoceptors, has been suggested from the results of behavioural studies of chronic pain and inflammation associated with arthritis (see Fitzgerald, 1989). For example, treatment with the non-selective $\beta$-adrenoceptor antagonist, propranolol, was found to decrease the signs and symptoms of inflammation in patients with rheumatoid arthritis (Kaplan et al., 1980). Similarly, it has been reported in animal studies that treatment with propranolol reduces the severity of joint injury in arthritic (Freunds adjuvant-induced) rats (Levine et al., 1988). Moreover, in this model of arthritis, $\alpha_1$, $\alpha_2$ or $\beta_1$-adrenoceptor antagonists were without effects, whereas the $\beta_2$-adrenoceptor antagonists, butoxamine or ICI 118551, were found to decrease the severity of joint injury (Levine et al., 1988). It has also been demonstrated that the $\beta_2$-adrenoceptor agonist, salbutamol, or the catecholamine, adrenaline (acting at $\beta_2$-adrenoceptors), exacerbate adjuvant-induced arthritis in the rat (Coderre et al., 1990; Coderre et al., 1991). Paradoxically, in other studies, $\beta_2$-adrenoceptor agonists have been reported to have anti-inflammatory actions. For example, $\beta_2$-adrenoceptor agonists such as salbutamol, and in particular

the longer-duration $\beta_2$-adrenoceptor agonist, salmeterol (Ball et al., 1991), are inhibitors of, mediator (histamine, leukotrienes and prostaglandins) release (Butchers et al., 1979; Butchers et al., 1991), bradykinin-induced plasma protein extravasation (Whelan et al., 1993), vascular permeability and granulocyte accumulation (Whelan & Johnson, 1992) and carrageenan-induced oedema (Green, 1972).

Electrophysiological recordings from C-fibre afferents have also suggested a role for adrenoceptors, particularly in inflamed tissues. For example, electrophysiological recordings from C-fibres in rabbit ears have shown that, although noradrenaline did not excite C-polymodal nociceptors in undamaged ears, approximately 60% of C-fibre nociceptors were excited by this catecholamine in ears with damaged auricular nerves (Sato & Perl, 1991). Noradrenaline-induced excitation was blocked by $\alpha_2$ (predominately) and $\alpha_1$-adrenoceptor antagonists; the effects of $\beta$ -adrenoceptor antagonists were not examined (Sato & Perl, 1991). In chronically lesioned nerves (neuromas) in the cat, adrenaline was shown to excite 12 of 30 unmyelinated skin afferents (Häbler et al., 1987), although the adrenoceptor mediating this action was not studied. In the investigation by Sanjue & Jun (1989), rat skin nociceptors showed no response to noradrenaline, although when there was a sustained neural discharge, induced by a compound algogenic substance (mixture of 5-HT, histamine, KCl and HCl), noradrenaline did enhance discharge in 7 of 9 afferents. The adrenoceptor responsible for this noradrenaline-induced excitation was not determined in this study. Noradrenaline-induced mechanical sensitisation has also been reported in rat cutaneous mechanonociceptors (Gold et al., 1994). Since such mechanical

sensitisation was blocked by yohimbine, this suggests the involvement of $\alpha_2$-adrenoceptors.

The aims of the present investigation were two-fold. Firstly, an electrophysiological study was performed in order to determine the effects of β-adrenoceptor agonists and antagonists and the catecholamines, adrenaline and noradrenaline, on neural discharge (spontaneous and mechanically-evoked) from fine articular afferents innervating normal rat ankle joints and in joints with Freunds adjuvant-induced monoarthritis. Secondly, behavioural studies, to examine the effects of β-adrenoceptor agonists and antagonists on the onset and progression of arthritis induced by Freunds adjuvant and their effects on rats with established localised adjuvant-arthritis.

## 6.2 MATERIALS & METHODS

### 6.2.1 Electrophysiological studies

The *in-vivo* preparation, neural recording, off-line analysis and statistical analysis are described in detail in Section 2. In brief, male Wistar rats (normal and adjuvant-arthritic) were anaesthetised with urethane and cannulations performed of the trachea, right carotid artery (blood pressure monitoring) and right femoral artery (retrograde cannulation for close intra-arterial bolus injections of drugs into the left limb). The medial aspect of the left ankle joint was exposed, and nerve fibres were isolated from the PACR nerve. C-fibre afferent discharge (spontaneous and mechanically-evoked) from articular mechanonociceptors was recorded extracellularly, using bipolar platinum-iridium electrodes.

**Protocol**

The protocol in these experiments involved an attempt to construct log dose-response curves to the $\beta_2$-adrenoceptor agonist, salbutamol (1-10μg i.a.). In a separate series of experiments, the effects on mechanonociceptor discharges of the long acting $\beta_2$ adrenoceptor agonist, salmeterol (5μg, i.a.), were examined. The effects of the non-selective β-adrenoceptor antagonist, propranolol, or of the selective $\beta_2$-adrenoceptor antagonist, ICI 118551 (1mgkg$^{-1}$, i.a.) were also determined. In some experiments, the catecholamines, adrenaline (10μg, i.a.) and noradrenaline (10μg, i.a.), were injected either before, or more typically, after injections of salbutamol (1 - 10μg) or salmeterol (5μg). Adrenaline (10μg) and noradrenaline (10μg) were also injected in the presence of propranolol (1mgkg$^{-1}$, i.a.).

**Data analysis**

In this series of experiments, if the test agent evoked an obvious change in spontaneous discharge then this was assessed by determining the number of impulses the test agent induced above or below the control discharge level (see Section 2.3.5.2 for further details of the formula used) with the delay and duration also being measured. If the test agent showed no clear response then the largest number of impulses both above and below basal discharge were determined by using an arbitrary time period of 60s over 0 - 20min post-injection of drug. The control period was defined as the 60s period immediately prior to the addition of the test substance. A significant change in spontaneous discharge was defined as a change over basal discharge of greater than 5 impulses for normal joints, and 10 impulses for arthritic joints. These values of 5 and 10 impulses were derived from the results of saline injections in normal ($3 \pm 1$ and $2 \pm 1$ impulses above and below basal discharge, respectively, 8 units) and arthritic ($5 \pm 4$ and $5 \pm 3$ impulses above and below basal discharge, respectively, 15 units) joints. Effects of the test substances on the responsiveness of the standard mechanical stimulus were assessed as the peak number of impulses above and / or below the pre-injection evoked discharge. A significant change in mechanically-evoked discharge was defined as a change of greater than 5 impulses for both normal and arthritic joints over the basal mechanically-evoked discharge. This value of 5 impulses was derived from the results of saline injections in normal ($2 \pm 1$ and $3 \pm 2$ impulses above and below basal discharge, respectively, 8 units) and arthritic ($3 \pm 2$ and $3 \pm 1$ impulses above and below basal discharge, respectively, 15 units) joints.

### 6.2.2 Behavioural studies

Two protocols were used in this series of behavioural experiments using the Freunds adjuvant model of arthritis, where the effects of injecting the $\beta_2$-adrenoceptor agonist, salmeterol, the non-selective β-adrenoceptor antagonist, propranolol, and the steroid, dexamethasone, were determined and compared to vehicle injections (the salmeterol vehicle was used: 99.9% phosphate buffer pH 7; 0.1% glacial acetic acid). In one protocol the effects of injecting (bolus injection i.p. once daily) test substances in rats with established (14 days post-adjuvant) localised adjuvant-induced arthritis of the left ankle joint were examined. In the other series of experiments, injections of test substances (bolus injection i.p. once daily) were started 2 days before the induction of adjuvant-arthritis. In both protocols, the following measurements were made: ankle joint circumference, and inflammation, mobility and pressure threshold scores (see Section 2.2.1 for details of these measurements). The various measurements were made approximately 30min after drug injections. Rat body weight was used as a general indicator of animal health. All measurements were made blind to drug treatment by the same experienced investigator.

## 6.3 RESULTS

### 6.3.1 *In-vivo* electrophysiology in normal and adjuvant-arthritic rat ankle joints

In this series of experiments, the effects of adrenoceptor agents were examined in 9 single units (8 experiments) from normal joints and in 21 single units (19 experiments) from adjuvant arthritic (22 ± 6 days post-adjuvant) joints. The mean afferent conduction velocity was in the C-fibre range for all the units recorded in normal (0.76 ± 0.23 $ms^{-1}$ ; range 0.3 - 1.5 $ms^{-1}$) and arthritic (0.83 ± 0.19 $ms^{-1}$; range 0.4 - 1.7 $ms^{-1}$) joints. No significant difference was found between the afferent conduction velocities in normal and arthritic joints ($P>0.05$, Mann Whitney U-test). Before the addition of any drugs, all units showed resting (spontaneous) discharge, although this was significantly greater in units from arthritic joints (3.1 ± 1.3 i.p.s.; range: 1.5 - 5.8 i.p.s.) than in those from normal joints (0.9 ± 0.3 i.p.s.; range: 0.2 - 1.7 i.p.s.) ($P<0.05$, Mann Whitney U-test). All the units examined were excited by close intra-arterial injection of capsaicin (1 - 3μg).

#### 6.3.1.1 Effects of adrenoceptor active drugs on mechanonociceptor discharge from normal joints

In all the units recorded from normal joints, the injection of the $\beta_2$-adrenoceptor agonists, salbutamol (1 - 10μg) and salmeterol (5μg) or the catecholamines, adrenaline (10μg) and noradrenaline (10μg), showed no significant effect (in comparison with saline injections) on spontaneous discharge (Table 6.1), nor in the responsiveness to the standard mechanical stimulus (Table 6.2). Similarly, the β-

**Table 6.1 Lack of effect of adrenoceptor agonists on spontaneous discharge from articular mechanonociceptors in normal rat ankle joints.**

| | | *units examined (n)* | *impulses above basal discharge* | *† P* | *impulses below basal discharge* | *† P* |
|---|---|---|---|---|---|---|
| saline | | 8 (8) | 3 ± 1 | - | 2 ± 1 | - |
| salbutamol | 1μg | 5 (4) | 2 ± 2 | NS | 2 ± 1 | NS |
| salbutamol | 3μg | 5 (4) | 3 ± 2 | NS | 3 ± 2 | NS |
| salbutamol | 10μg | 8 (8) | 4 ± 1 | NS | 1 ± 2 | NS |
| salmeterol | 5μg | 3 (3) | 2 ± 1 | NS | 3 ± 1 | NS |
| adrenaline | 10μg | 8 (7) | 2 ± 2 | NS | 2 ± 2 | NS |
| noradrenaline | 10μg | 8 (7) | 2 ± 2 | NS | 1 ± 2 | NS |

† Statistical comparisons (Mann Whitney U-test) between saline and the test adrenergic drug. NS = P>0.05. The pre-injection discharge, before the injection of any drugs, was 0.9 ± 0.3 i.p.s.

**Table 6.2 Lack of effect of adrenoceptor agonists on mechanically-evoked discharge from articular mechanonociceptors in normal rat ankle joints.**

| | | *units examined (n)* | *impulses above evoked basal discharge* | *† P* | *impulses below evoked basal discharge* | *† P* |
|---|---|---|---|---|---|---|
| saline | | 8 (8) | 1 ± 2 | - | 3 ± 2 | - |
| salbutamol | 1μg | 4 (4) | 3 ± 2 | NS | 2 ± 1 | NS |
| salbutamol | 3μg | 4 (4) | 2 ± 1 | NS | 2 ± 3 | NS |
| salbutamol | 10μg | 8 (8) | 1 ± 2 | NS | 1 ± 2 | NS |
| salmeterol | 5μg | 3 (3) | 3 ± 1 | NS | 1 ± 3 | NS |
| adrenaline | 10μg | 8 (7) | 2 ± 2 | NS | 1 ± 2 | NS |
| noradrenaline | 10μg | 8 (7) | 2 ± 2 | NS | 3 ± 2 | NS |

† Statistical comparisons (Mann Whitney U-test) between saline and the test adrenergic drug. NS = P>0.05. The pre-injection mechanically-evoked discharge, before the injection of any drugs, was 38 ± 9 impulses.

adrenoceptor antagonist, propranolol (1mgkg$^{-1}$, i.a.), had no significant effect on either spontaneous (Table 6.3) or mechanically- evoked discharge (Table 6.4).

#### 6.3.1.2 Effects of $\beta_2$-adrenoceptor agonists on mechanonociceptor discharge from arthritic joints

The effects of the $\beta_2$-adrenoceptor agonist, salbutamol (1 - 10μg), was examined in 14 units (13 experiments) recorded from arthritic joints. Of these, 11 units (10 experiments) showed no significant change in either spontaneous discharge (Figure 6.1) or in the response to the standard mechanical stimulus (Figure 6.2). However, in the remaining 3 units (3 experiments) a significant increase in spontaneous discharge (excitation response) was produced by the high dose of salbutamol (10μg) (see Figure 6.3 for typical response and pooled data in Figure 6.4). This salbutamol-induced excitation had a delay to onset of 140 ± 20s and a response duration of 45 ± 10s, and was antagonised by the β-adrenoceptor antagonist, propranolol (1mgkg$^{-1}$, i.a.) (Figure 6.4). None of the 3 units excited by salbutamol showed any significant effects on the responsiveness to the mechanical stimulus ($P>0.05$, Mann Whitney U-test, versus saline). The long-acting $\beta_2$-adrenoceptor agonist, salmeterol (5μg), did not cause any significant change in either spontaneous (Figure 6.5) or mechanically-evoked (Figure 6.6) discharge in any of the 6 units (5 experiments) recorded. Although salmeterol (5μg) produced no change in afferent neural discharge, it did cause a prolonged fall in arterial blood pressure (see Figure 6.7 for a typical response).

**Table 6.3 Lack of effect of propranolol on spontaneous discharge from articular mechanonociceptors in normal rat ankle joints.**

| | *units examined (n)* | *impulses above basal discharge* | *† P* | *impulses below basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 8 (8) | 3 ± 1 | - | 2 ± 1 | - |
| propranolol 1mgkg$^{-1}$ | 8 (8) | 3 ± 2 | NS | 2 ± 3 | NS |

† Statistical comparisons (Mann Whitney U-test) between saline and propranolol. NS = P>0.05. The pre-injection discharge, was 0.9 ± 0.3 i.p.s pre-saline and 0.8 ± 0.4 i.p.s. pre-propranolol.

**Table 6.4 Lack of effects of propranolol on mechanically-evoked discharge from articular mechanonociceptors in normal rat ankle joints.**

| | *units examined (n)* | *impulses above evoked basal discharge* | *† P* | *impulses below evoked basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 7 (7) | 2 ± 1 | - | 3 ± 2 | - |
| propranolol 1mg kg$^{-1}$ | 7 (7) | 3 ± 2 | NS | 2 ± 2 | NS |

† Statistical comparisons (Mann Whitney U-test) between saline and propranolol. NS = P>0.05. The pre-injection mechanically-evoked discharge, was 38 ± 9 impulses pre-saline and 28 ± 8 impulses pre-propranolol.

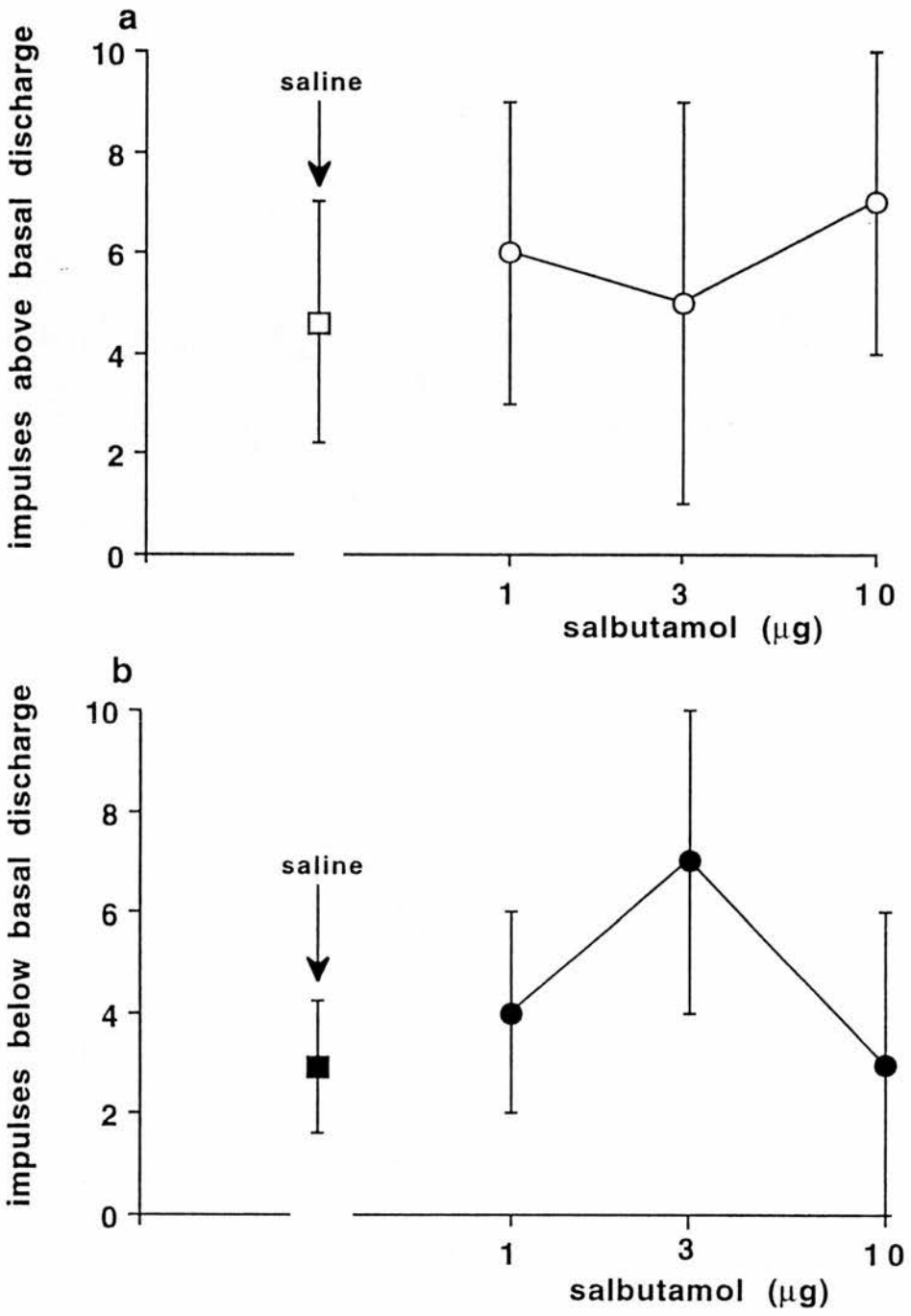



Figure 6.1 Absence of either (a) enhancement or (b) depression by salbutamol (1 - 10μg) of spontaneous discharge from mechanonociceptors in chronically arthritic (15-23 days post-adjuvant) joints. The pre-injection discharge, was 2.9 ± 1.3 i.p.s. pre-saline and 3.2 ± 1.6 i.p.s. pre-salbutamol. Each point is the mean ± s.e.mean from 11 units (10 experiments).

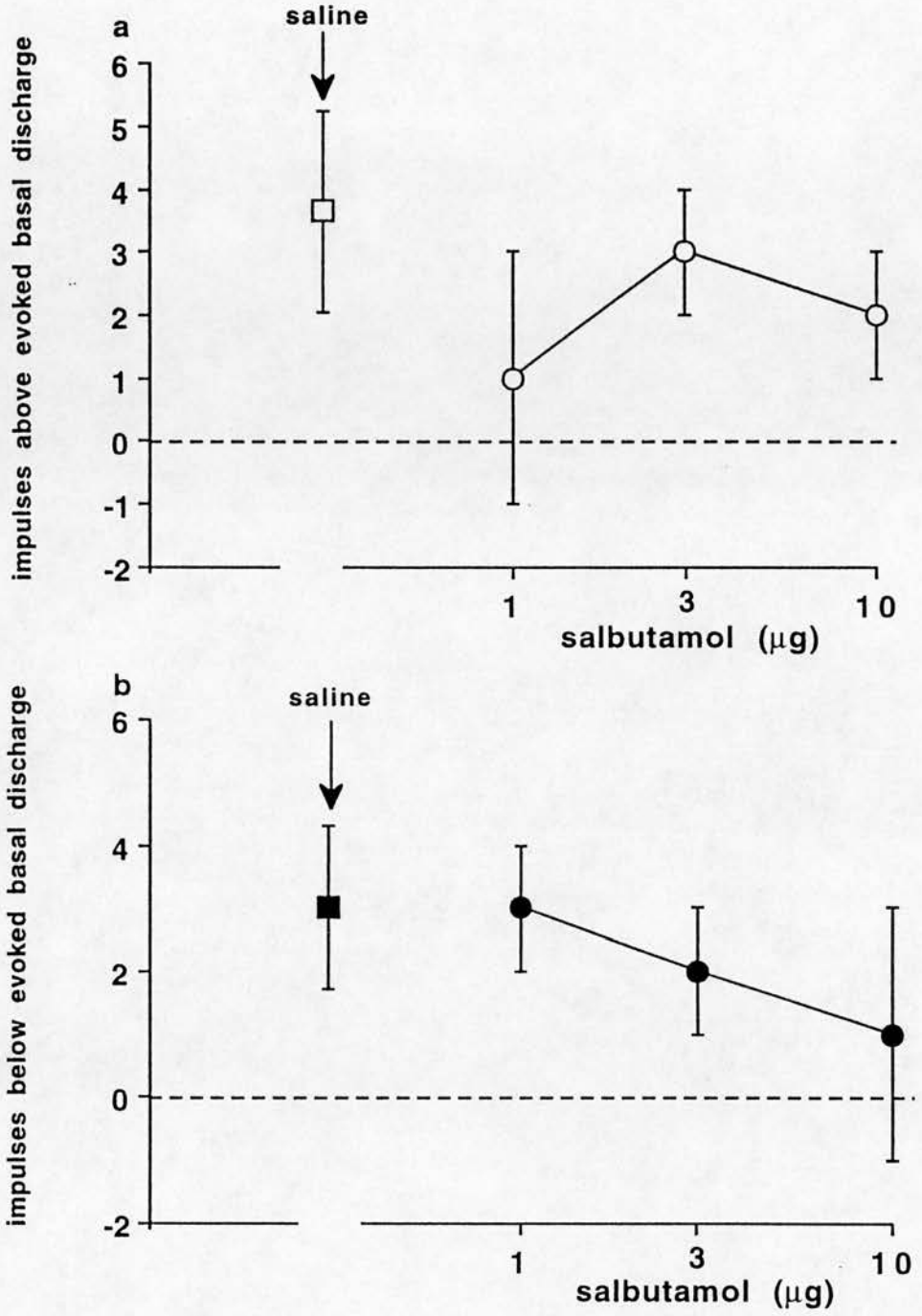
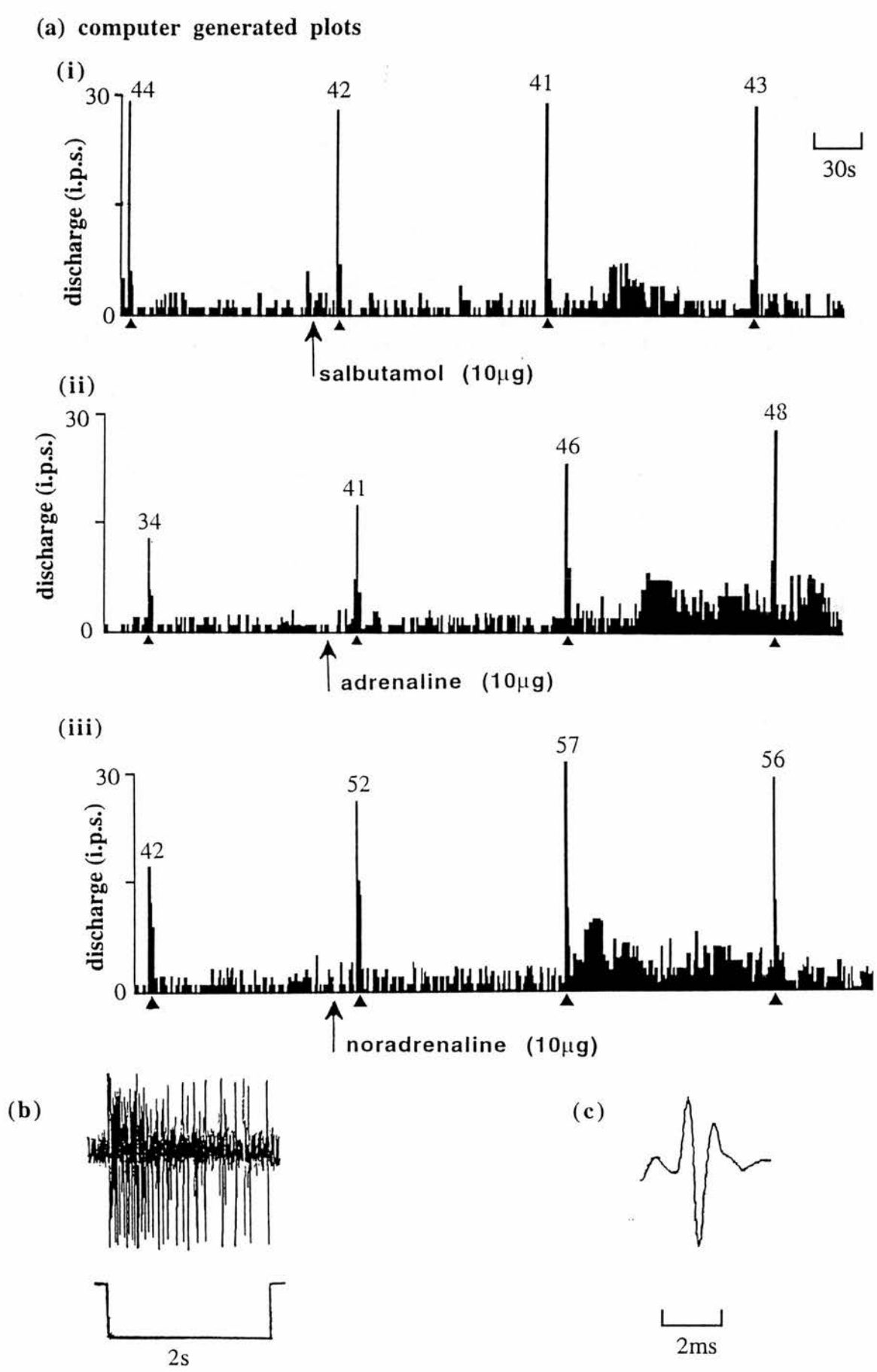



Figure 6.2 Absence of either (a) enhancement or (b) depression by salbutamol (1 - 10μg) of mechanically-evoked discharge from mechanonociceptors in chronically arthritic (15-23 days post-adjuvant) joints. The pre-injection mechanically-evoked discharge, was 49 ± 10 impulses pre-saline and 47 ± 12 impulses pre-salbutamol. Each point is the mean ± s.e.mean from 11 units (10 experiments).


(a) computer generated plots
(i)
30
0
discharge (i.p.s.)
44
42
41
43
30s
salbutamol (10μg)
(ii)
30
0
discharge (i.p.s.)
34
41
46
48
adrenaline (10μg)
(iii)
30
0
discharge (i.p.s.)
42
52
57
56
noradrenaline (10μg)
(b)
2s
(c)
2ms

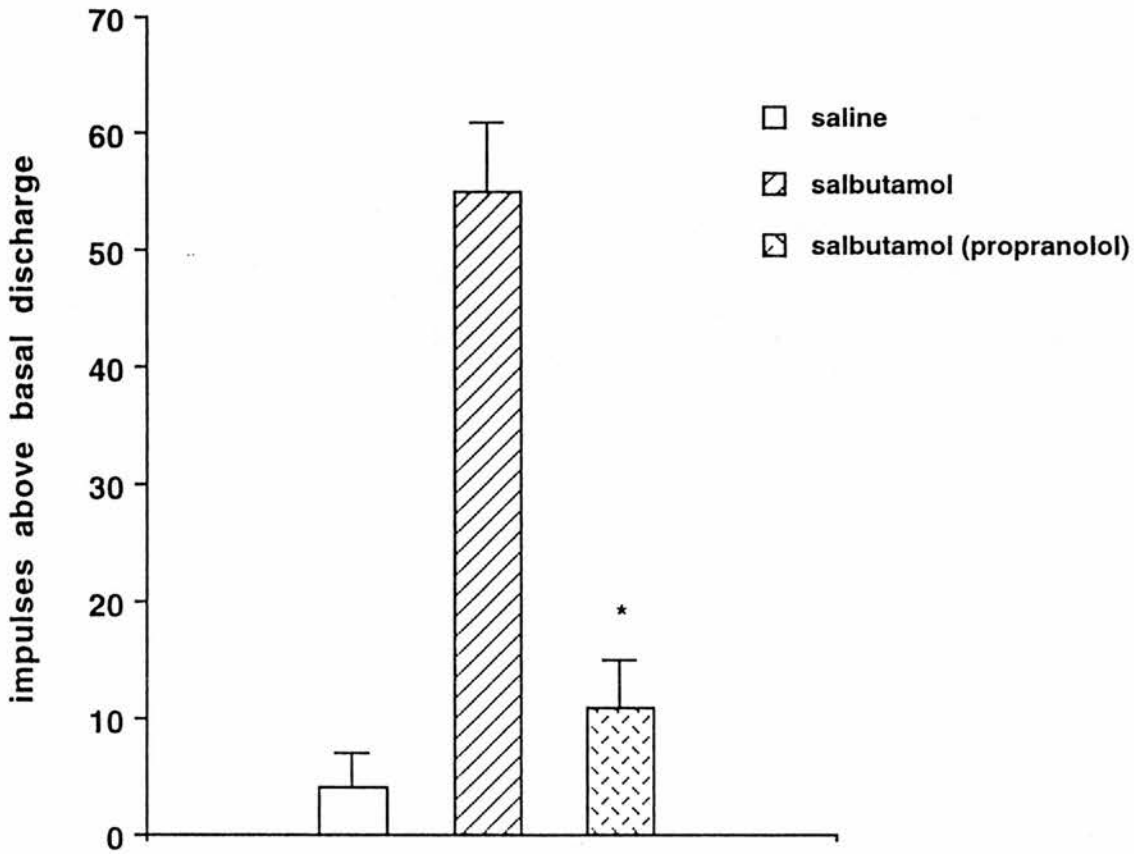



Figure 6.4 Salbutamol (10μg)-induced enhancement, in the absence and presence of propranolol (1mgkg$^{-1}$, i.a.), of spontaneous discharge from mechanonociceptors in chronically arthritic (18 - 30 days post-adjuvant) rat ankle joints (pre-injection discharge was 2.5 ± 1.2 i.p.s. pre-saline; 2.6 ± 1.4 i.p.s. pre-salbutamol; 2.9 ± 0.8 i.p.s. pre-salbutamol in the presence of propranolol). Each point is the mean ± s.e.mean from 3 units (3 experiments). * $P<0.05$, paired t-test, versus salbutamol.

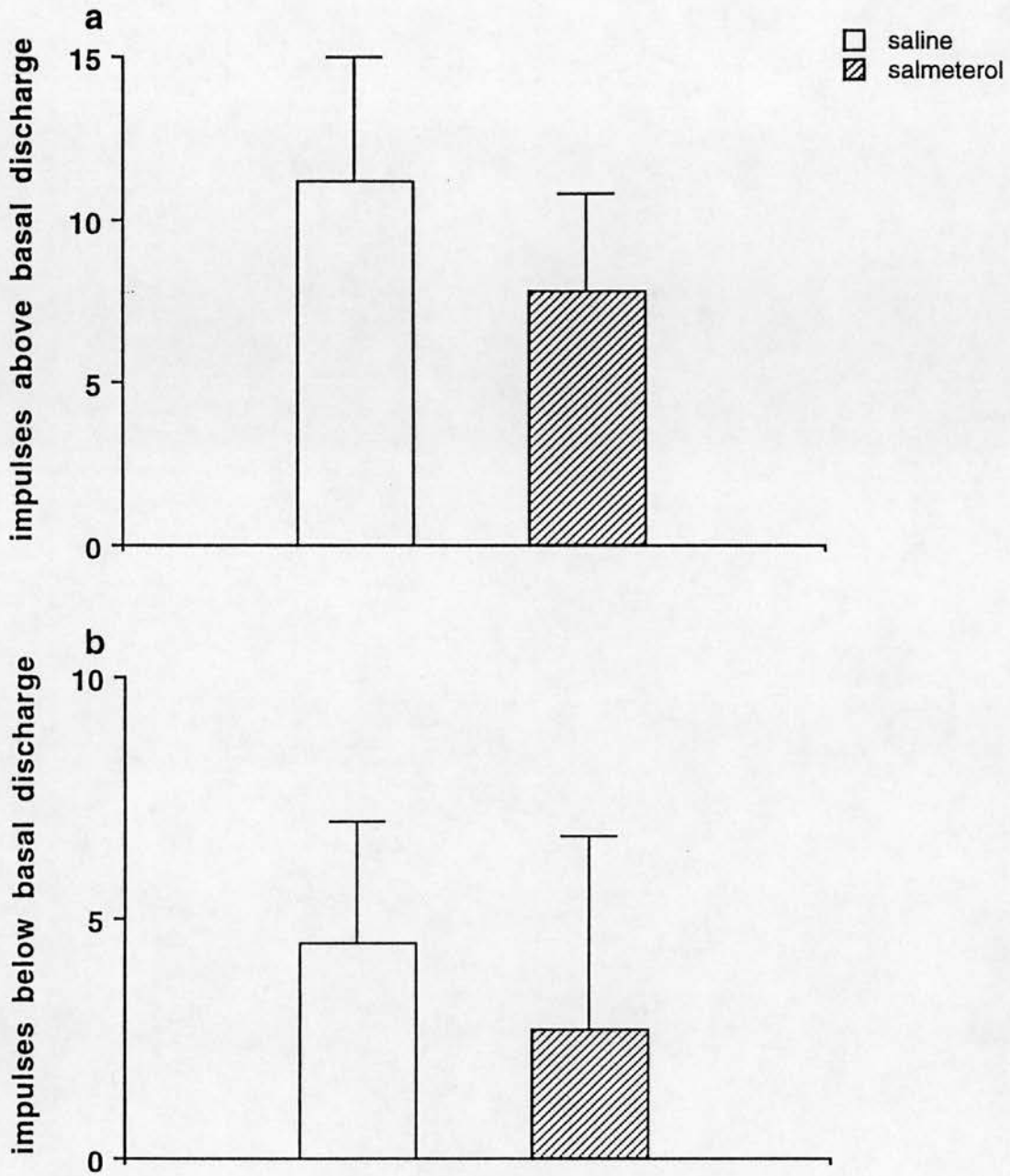



Figure 6.5 Absence of either (a) enhancement or (b) depression by salmeterol (5μg) of spontaneous discharge from mechanonociceptors in chronically arthritic (15-24 days post-adjuvant) joints ( pre-injection discharge was 3.3 ± 0.8 i.p.s. pre-saline; 3.4 ± 1.3 i.p.s. pre-salmeterol). Each point is the mean ± s.e.mean from 6 units (5 experiments).

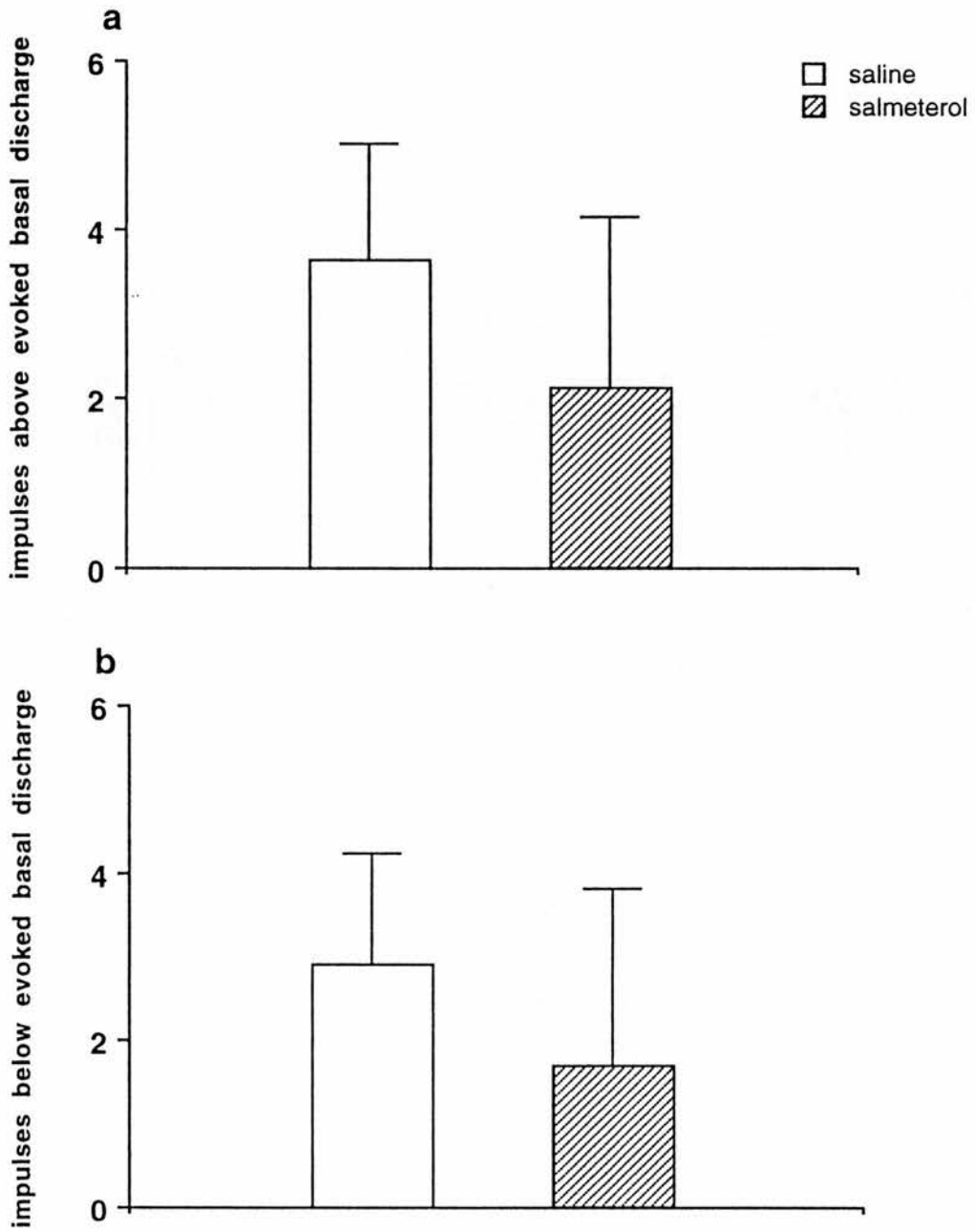



Figure 6.6 Absence of either (a) enhancement or (b) depression by salbutamol (5µg) of mechanically-evoked discharge from mechanonociceptors in chronically arthritic (15-24 days post-adjuvant) joints (pre-injection mechanically-evoked discharge was 50 ± 13 impulses pre-saline; 42 ± 11 impulses pre-salmeterol). Each point is the mean ± s.e.mean from 5 units (4 experiments).

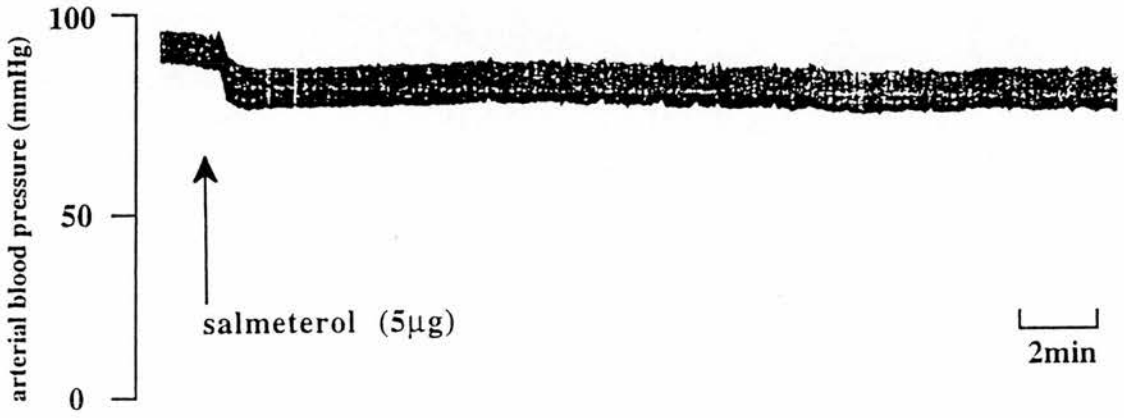



Figure 6.7 Chart recording showing the typical long-lasting hypotension associated with intra-arterial injection of salmeterol (5μg).

#### 6.3.1.3 Effects of β-adrenoceptor antagonists on mechanonociceptor discharge from arthritic joints

In none of the units recorded, did either the non-selective β-adrenoceptor antagonist, propranolol (1mgkg$^{-1}$, i.a.), or the selective $\beta_2$-adrenoceptor antagonist, ICI 118551 (1mgkg$^{-1}$, i.a.), have any significant effect on either spontaneous (Table 6.5) or mechanically- evoked (Table 6.6) discharge.

#### 6.3.1.4 Effects of adrenaline and noradrenaline on mechanonociceptor discharge from arthritic joints

##### 6.3.1.4.1 Effects of adrenaline and noradrenaline on spontaneous discharge from arthritic joints

The effects of the catecholamines, adrenaline (10μg, i.a.) and noradrenaline (10μg, i.a.), on spontaneous discharge are summarised in Table 6.7. Of the 14 units (13 experiments) tested with adrenaline (10μg), 7 units (7 experiments) showed an excitatory response (see Figure 6.3 for typical response) with the remaining units having no effect. The effects of noradrenaline (10μg) were examined on 12 units (11 experiments). Of these units, noradrenaline excited (see Figure 6.3 for typical response) 7 units (7 experiments) with the remaining units showing no change in spontaneous discharge. The excitation evoked by adrenaline or noradrenaline was unaffected by the β-adrenoceptor antagonist, propranolol (1mgkg$^{-1}$, i.a.)(Table 6.7). The excitations induced by adrenaline (10μg) and noradrenline (10μg) were significantly greater (approximately 2 and 2.5 fold, respectively) than the excitation evoked by salbutamol (10μg, 3 units) (Table 6.7). Moreover, the duration of the

**Table 6.5 Lack of effect of β-adrenoceptor antagonists on spontaneous discharge from articular mechanonociceptors in arthritic rat ankle joints.**

| | *units examined (n)* | *impulses above basal discharge* | *† P* | *impulses below basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 15 (14) | 5 ± 4 | - | 5 ± 3 | - |
| propranolol 1mgkg$^{-1}$ | 9 (8) | 6 ± 3 | NS | 3 ± 2 | NS |
| ICI 118551 1mgkg$^{-1}$ | 4 (4) | 6 ± 4 | NS | 7 ± 3 | NS |

† Statistical comparisons (Mann Whitney U-test) between saline and the test β-adrenoceptor antagonist. NS = P>0.05. The pre-injection discharge, was 3.1 ± 1.3 i.p.s pre-saline, 3.4 ± 1.5 i.p.s. pre-propranolol and 2.7 ± 1.4 i.p.s. pre-ICI 118551.

**Table 6.6 Lack of effect of β -adrenoceptor antagonists on mechanically-evoked discharge from articular mechanonociceptors in arthritic rat ankle joints.**

| | *units examined (n)* | *impulses above evoked basal discharge* | *† P* | *impulses below evoked basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 15 (14) | 3 ± 2 | - | 3 ± 1 | - |
| propranolol 1mg kg$^{-1}$ | 9 (8) | 3 ± 1 | NS | 1 ± 1 | NS |
| ICI 118551 1mgkg$^{-1}$ | 4 (4) | 3 ± 2 | NS | 2 ± 2 | NS |

† Statistical comparisons (Mann Whitney U-test) between saline and the test β-adrenoceptor antagonist. NS = P>0.05. The pre-injection mechanically-evoked discharge, was 49 ± 9 impulses pre-saline, 32 ± 13 impulses pre-propranolol and 38 ± 8 impulses pre-ICI 118551.

**Table 6.7 Enhancement of spontaneous discharge from mechanonociceptors by salbutamol, adrenaline and noradrenaline in the (a) absence and (b) presence of propranolol in arthritic rat ankle joints.**

**a) absence of propranolol**

| | | *units examined (n)* | *units excited (n)* | *impulses above basal discharge* ‡ | *delay (s)* ‡ | *duration (s)* ‡ |
|---|---|---|---|---|---|---|
| salbutamol | 10μg | 14 (13) | 3 (3) | 55 ± 6 | 140 ± 20 | 45 ± 10 |
| adrenaline | 10μg | 14 (13) | 7 (7) | 90 ± 29 * | 146 ± 50 | 78 ± 11 * |
| noradrenaline | 10μg | 12 (11) | 7 (7) | 141 ± 40 * | 124 ± 36 | 94 ± 17 * |

* indicates a statistically significant ($P<0.05$, Mann Whitney U-test) difference as compared to salbutamol. The pre-injection discharge, was 2.5 ± 1.2 i.p.s pre-salbutamol, 2.7 ± 1.4 i.p.s. pre-adrenaline and 2.9 ± 0.8 i.p.s. pre-noradrenaline. ‡ these values refer only to the units that were excited.

**b) presence of propranolol (1mgkg$^{-1}$ ,i.a.)**

| | | *units examined* | *units excited* | *impulses above basal discharge* ‡ | *delay (s)* ‡ | *duration (s)* ‡ |
|---|---|---|---|---|---|---|
| salbutamol | 10μg | 3 (3) | 3 (3) | 11± 4 † | 107 ± 20 | 25 ± 17 |
| adrenaline | 10μg | 7 (7) | 7 (7) | 111± 38 | 77 ± 23 | 84 ± 14 |
| noradrenaline | 10μg | 7 (7) | 7 (7) | 100 ± 43 | 112 ± 28 | 80 ± 10 |

† indicates a statistically significant ($P<0.05$, Mann Whitney U-test) difference as compared to the value in the absence of propranolol (see Table 6.7a). The pre-injection discharge, was 2.7 ± 1.4 i.p.s pre-salbutamol, 2.9 ± 0.8 i.p.s. pre-adrenaline and 2.9 ± 0.9 i.p.s. pre-noradrenaline. ‡ these values refer only to the units that were excited.

excitation induced by adrenaline (10μg) or noradrenline (10μg) was approximately two-fold greater than that induced by salbutamol (10μg, 3 units) (Table 6.7).

#### 6.3.1.4.2 Effects of adrenaline and noradrenaline on mechanically-evoked discharge from arthritic joints

Table 6.8 summarizes the effects of adrenaline (10μg) and noradrenaline (10μg) on the responsiveness to the standard mechanical stimulus. In approximately half of the units recorded, adrenaline (10μg) and noradrenaline (10μg) enhanced (sensitised) the response to the mechanical stimulus (see Figure 6.3 for typical responses). All units that were sensitised by adrenaline (10μg) were also sensitised by noradrenaline (10μg). In all the units tested, the sensitisations induced by adrenaline (10μg) and noradrenaline (10μg) were unaffected by the β-adrenoceptor antagonist, propranolol ($1 mg kg^{-1}$, i.a.) (Table 6.8).

### 6.3.2 Behavioural studies using rats with adjuvant-induced arthritis

#### 6.3.2.1 Influence of salmeterol on an established localised adjuvant-induced arthritis in the rat

In rats with an established localised adjuvant-induced arthritis of the left ankle joint, the selective $\beta_2$-adrenoceptor agonist, salmeterol ($50 \mu g kg^{-1}$, i.p.), caused no significant change (compared with vehicle injections) in any of the parameters measured. Thus, left ankle joint circumference (Figure 6.8), left hind limb withdrawal pressure score (Figure 6.9), left walking foot placement score (Figure 6.10), left ankle joint inflammation score (Figure 6.11) and rat body weight (Figure 6.12) were all

**Table 6.8 Enhancement of mechanically-evoked discharge from mechanonociceptors by adrenaline and noradrenaline in the (a) absence and (b) presence of propranolol in arthritic rat ankle joints.**

**a) absence of propranolol**

| | | *units examined (n)* | *units excited (n)* | *impulses above basal discharge* ‡ |
|---|---|---|---|---|
| adrenaline | 10μg | 14 (13) | 7 (7) | 17 ± 3 |
| noradrenaline | 10μg | 12 (11) | 7 (7) | 13 ± 2 |

The pre-injection mechanically-evoked discharge, was 43 ± 15 impulses pre-adrenaline and 54 ± 16 impulses pre-noradrenaline. ‡ these values refer only to the units that were excited.

**b) presence of propranolol (1mgkg$^{-1}$, i.a.)**

| | | *units examined (n)* | *units excited (n)* | *impulses above basal discharge* ‡ | *† P* |
|---|---|---|---|---|---|
| adrenaline | 10μg | 4 (4) | 4 (4) | 16 ± 3 | NS |
| noradrenaline | 10μg | 4 (4) | 4 (4) | 12 ± 3 | NS |

† Statistical comparison (Mann Whitney U-test) between values obtained in the absence of propranolol against those in its presence (see Table 6.8a). NS = P>0.05. The pre-injection mechanically-evoked discharge in the presence of propranolol, was 36 ± 10 impulses pre-adrenaline and 37 ± 10 impulses pre-noradrenaline. ‡ these values refer only to the units that were excited.

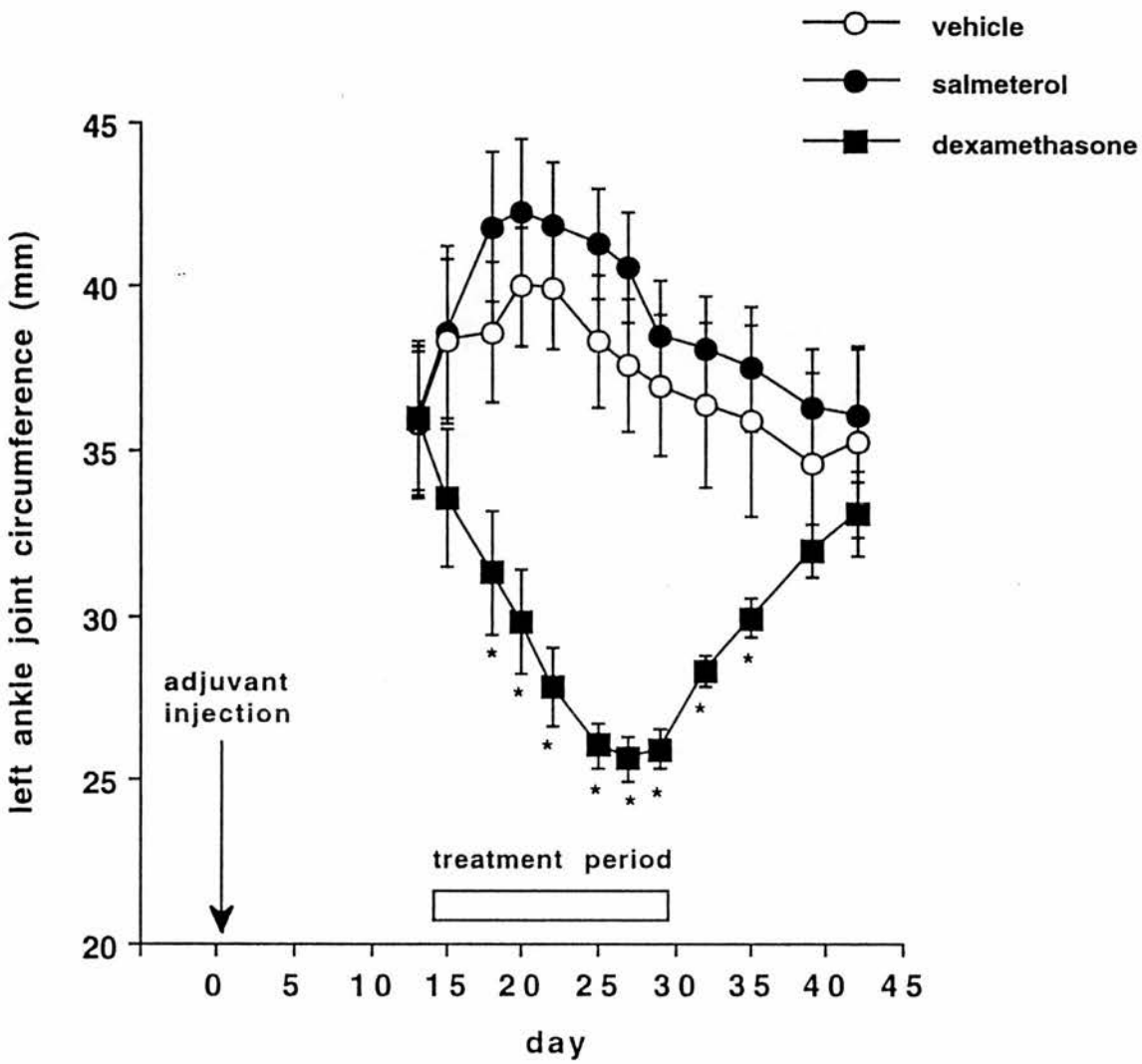



Figure 6.8 Effects of vehicle, salmeterol (50μgkg$^{-1}$, i.p.) and dexamethasone (200μgkg$^{-1}$, i.p.) on the circumference of adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: $P<0.05$, ANOVA days 14-29. * $P<0.05$, Mann Whitney U-test, versus vehicle.

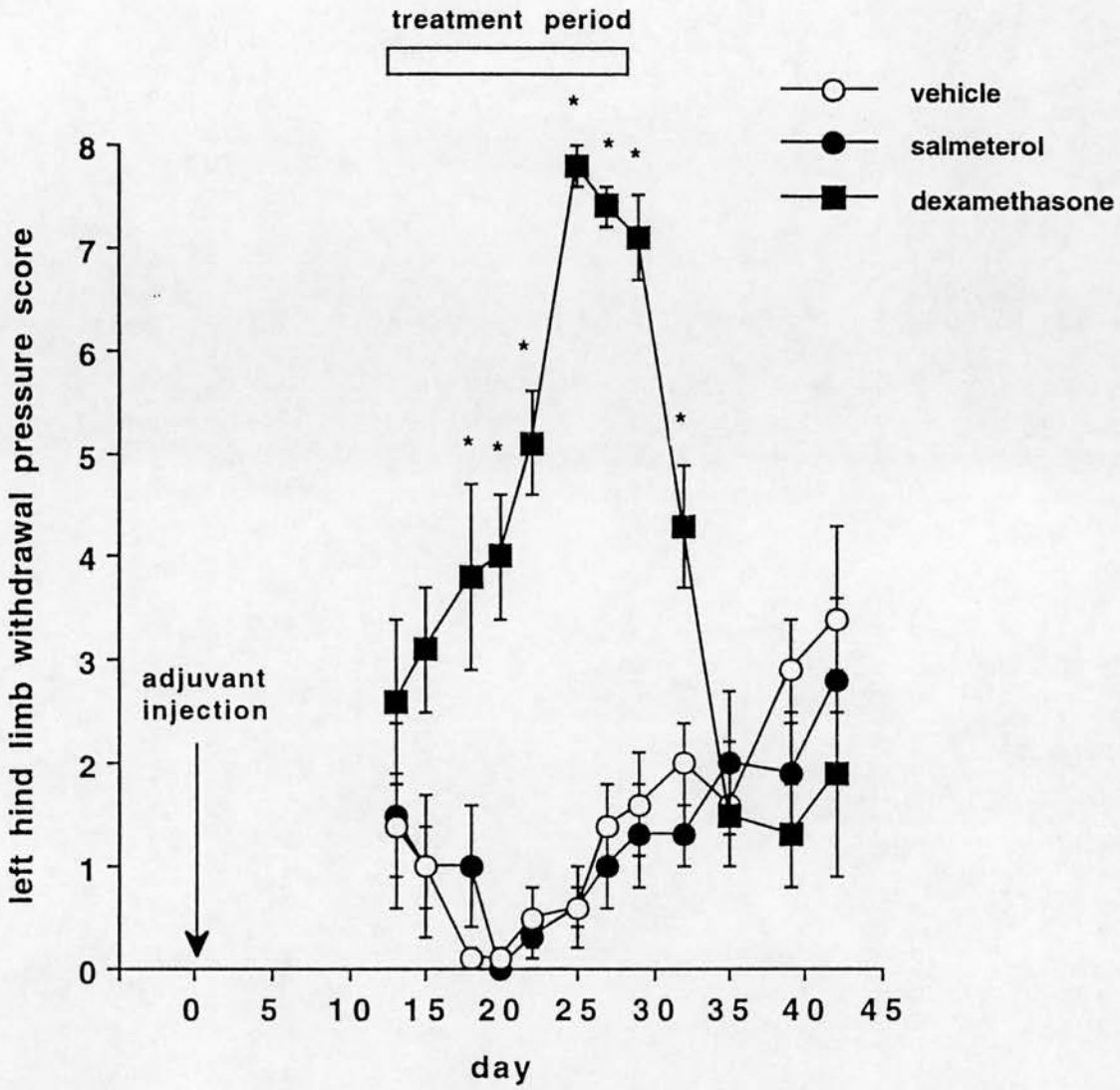



Figure 6.9 Effects of vehicle, salmeterol (50μgkg$^{-1}$, i.p.) and dexamethasone (200μgkg$^{-1}$, i.p.) on the withdrawal thresholds of adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: $P<0.05$ ANOVA days 14-29. * $P<0.05$, Mann Whitney U-test, versus vehicle.

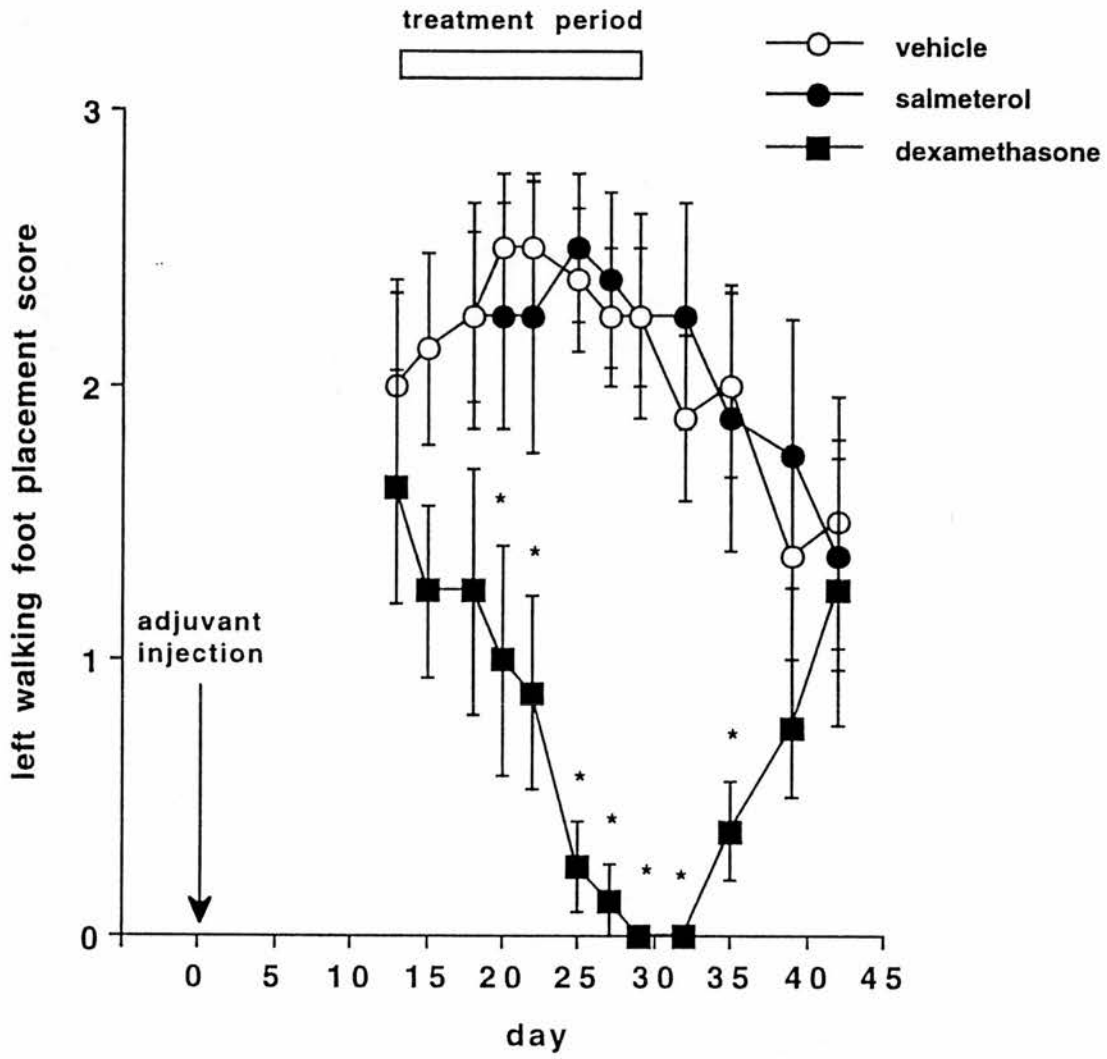



Figure 6.10 Effects of vehicle, salmeterol (50μgkg$^{-1}$, i.p.) and dexamethasone (200μgkg$^{-1}$, i.p.) on left walking foot placement scores in rats with adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: * P<0.05, Mann Whitney U-test, versus vehicle.

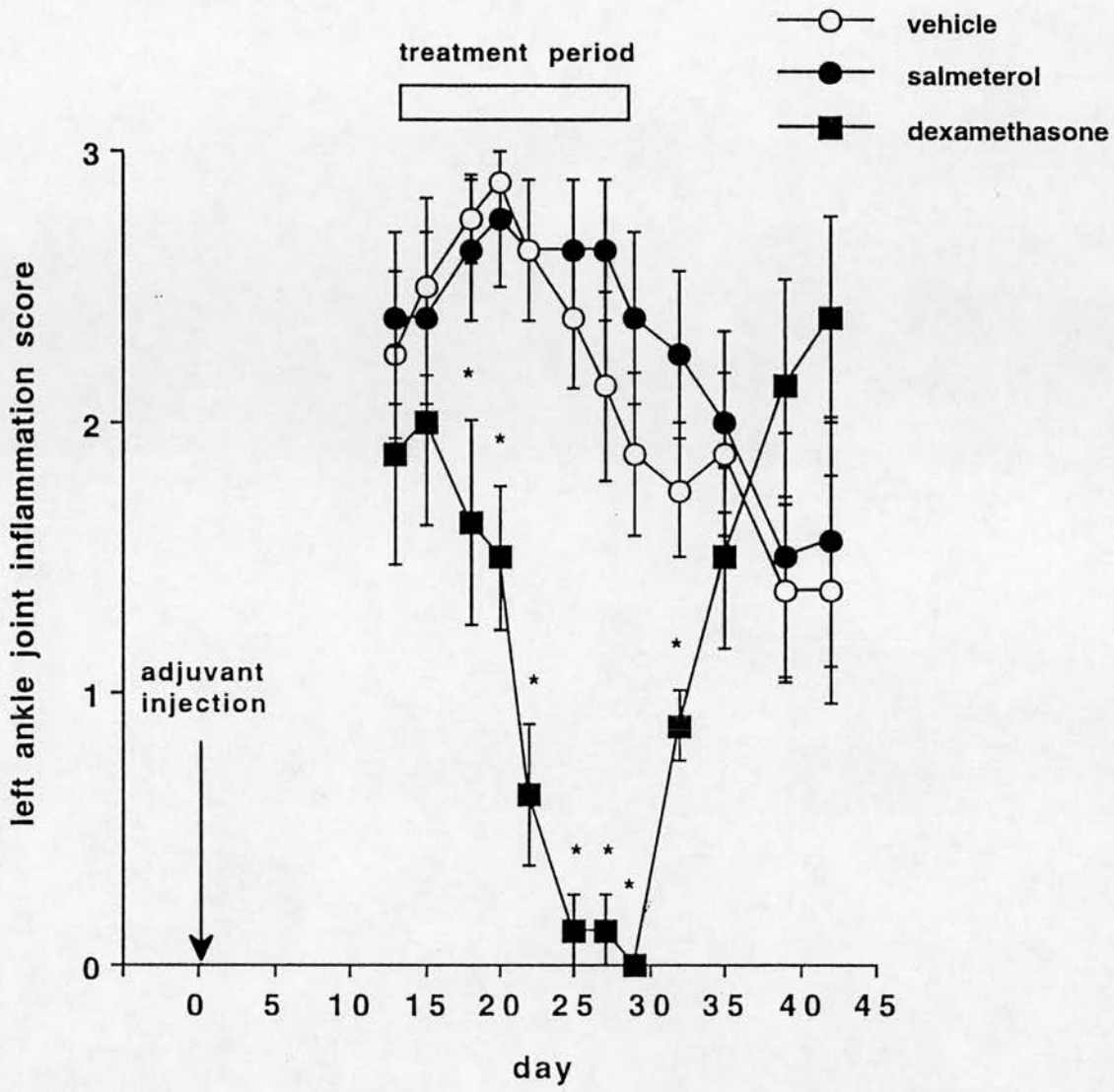



Figure 6.11 Effects of vehicle, salmeterol (50μgkg$^{-1}$, i.p.) and dexamethasone (200μgkg$^{-1}$, i.p.) on inflammation scores of adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: * $P<0.05$, Mann Whitney U-test, versus vehicle.

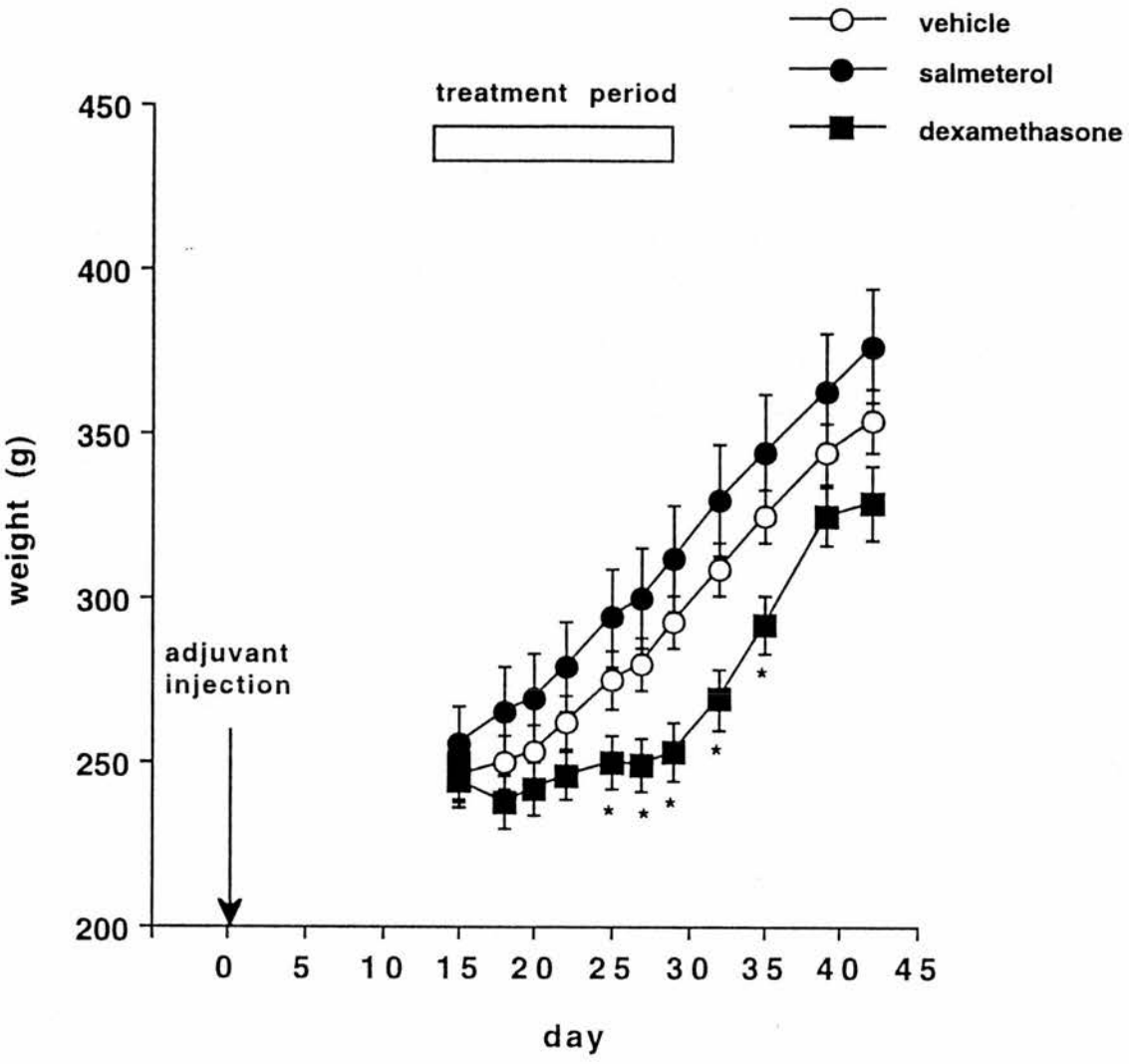



Figure 6.12 Effects of vehicle, salmeterol ($50\mu gkg^{-1}$, i.p.) and dexamethasone ($200\mu gkg^{-1}$, i.p.) on weight of rats with adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: $P<0.05$ ANOVA days 14-29. * $P<0.05$, Mann Whitney U-test, versus vehicle.

unchanged. A higher dose of salmeterol (150μgkg$^{-1}$, i.p.) also had no significant effect ($P>0.05$, Mann Whitney U-test, versus vehicle) in any of the parameters examined (for clarity of Figures, data is not shown). In contrast, dexamethasone (200μgkg$^{-1}$, i.p.), caused significant changes in all of the parameters measured (Figures 6.8 -6.12). Once dexamethasone treatment was terminated there was an increase in left ankle joint circumference (Figure 6.8), a decrease in left hind limb withdrawal pressure score (Figure 6.9) and increases in left walking foot placement score (Figure 6.10), left ankle joint inflammation score (Figure 6.11) and rat body weight (Figure 6.12) to such an extent that the parameters did not differ significantly (5 - 10 days after terminating treatment) from those of the vehicle-treated rats.

#### 6.3.2.2 Influence of salmeterol and propranolol on the development of a localised adjuvant-induced arthritis in the rat

Intra-peritoneal injections of the selective $\beta_2$-adrenoceptor agonist, salmeterol (50μgkg$^{-1}$), or of the β-adrenoceptor antagonist, propranolol (1mgkg$^{-1}$), caused no significant change (compared to vehicle injections) in the development of either the acute (day 2 - 9) or chronic (day 9 - 19) phases of arthritis (Freunds adjuvant injected day 1); there was no change in left ankle joint circumference (Figure 6.13), left hind limb withdrawal pressure score (Figure 6.14), left walking foot placement score (Figure 6.15), left ankle joint inflammation score (Figure 6.16) and rat body weight (Figure 6.17). In contrast, treatment with dexamethasone (200μgkg$^{-1}$, i.p.), showed significant changes in all of these parameters (Figures 6.13 -6.17) in both the acute and chronic phases of the arthritis. Once dexamethasone treatment was

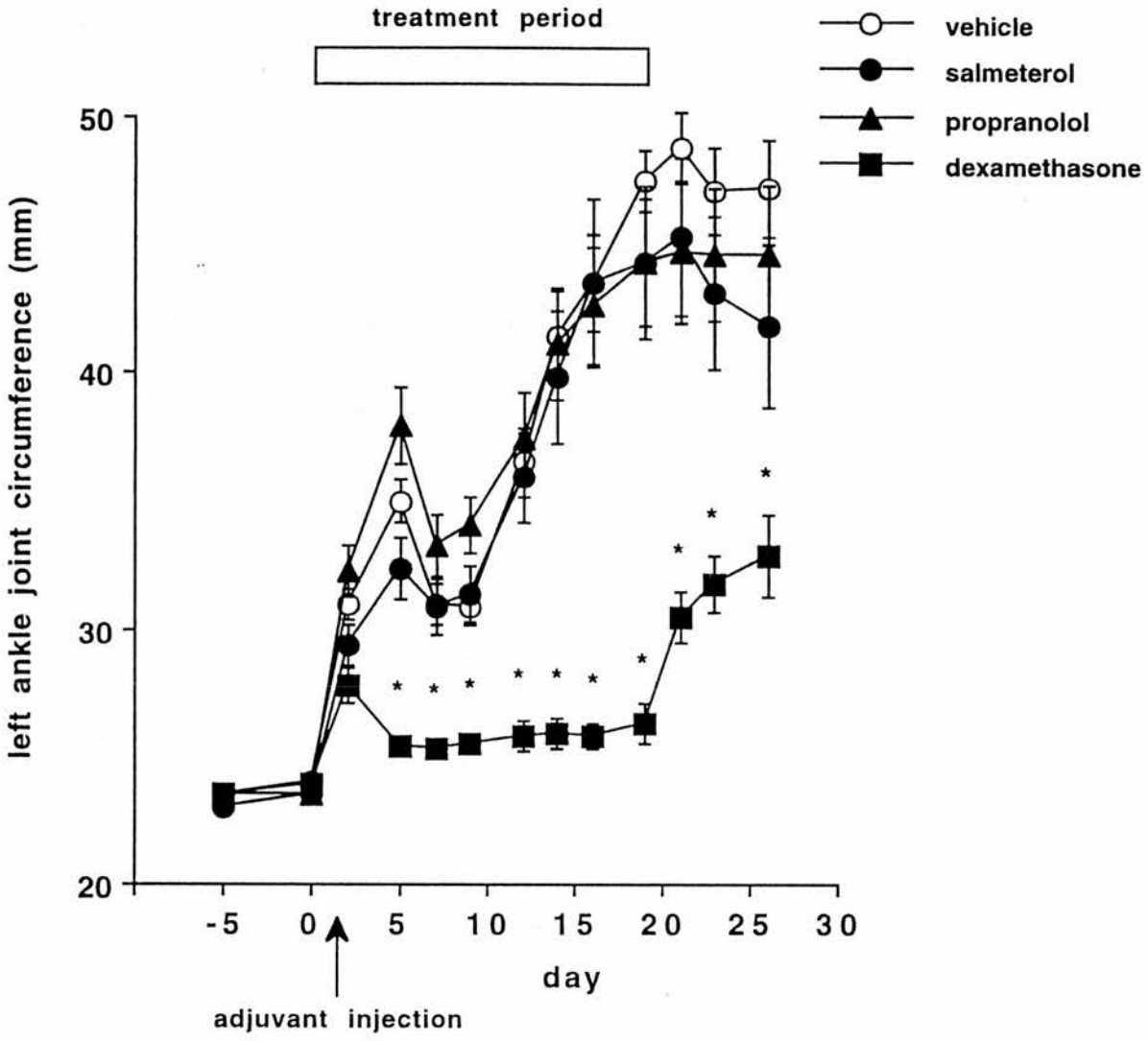



Figure 6.13 Effects of vehicle, salmeterol (50μgkg$^{-1}$, i.p.), propranolol (1mgkg$^{-1}$, i.p.) and dexamethasone (200μgkg$^{-1}$, i.p.) on the circumference of adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: $P<0.05$, ANOVA days 2-9 and days 9-19. * $P<0.05$, Mann Whitney U-test, versus vehicle.

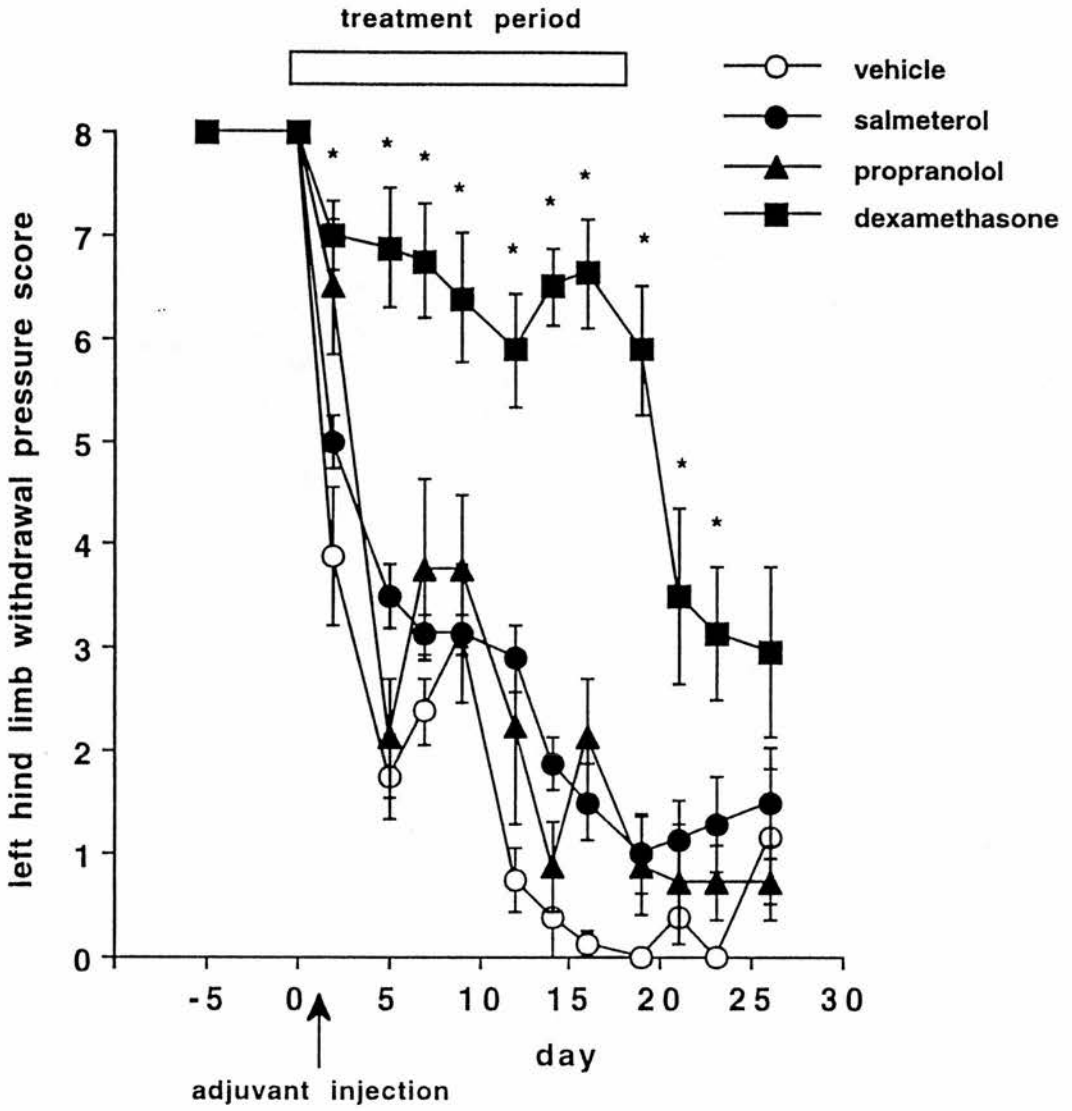



Figure 6.14 Effects of vehicle, salmeterol (50μgkg$^{-1}$, i.p.), propranolol (1mgkg$^{-1}$, i.p.) and dexamethasone (200μgkg$^{-1}$, i.p.) on the withdrawal thresholds of adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: $P<0.05$ ANOVA days 2-9 and 9-19. * $P<0.05$, Mann Whitney U-test, versus vehicle.

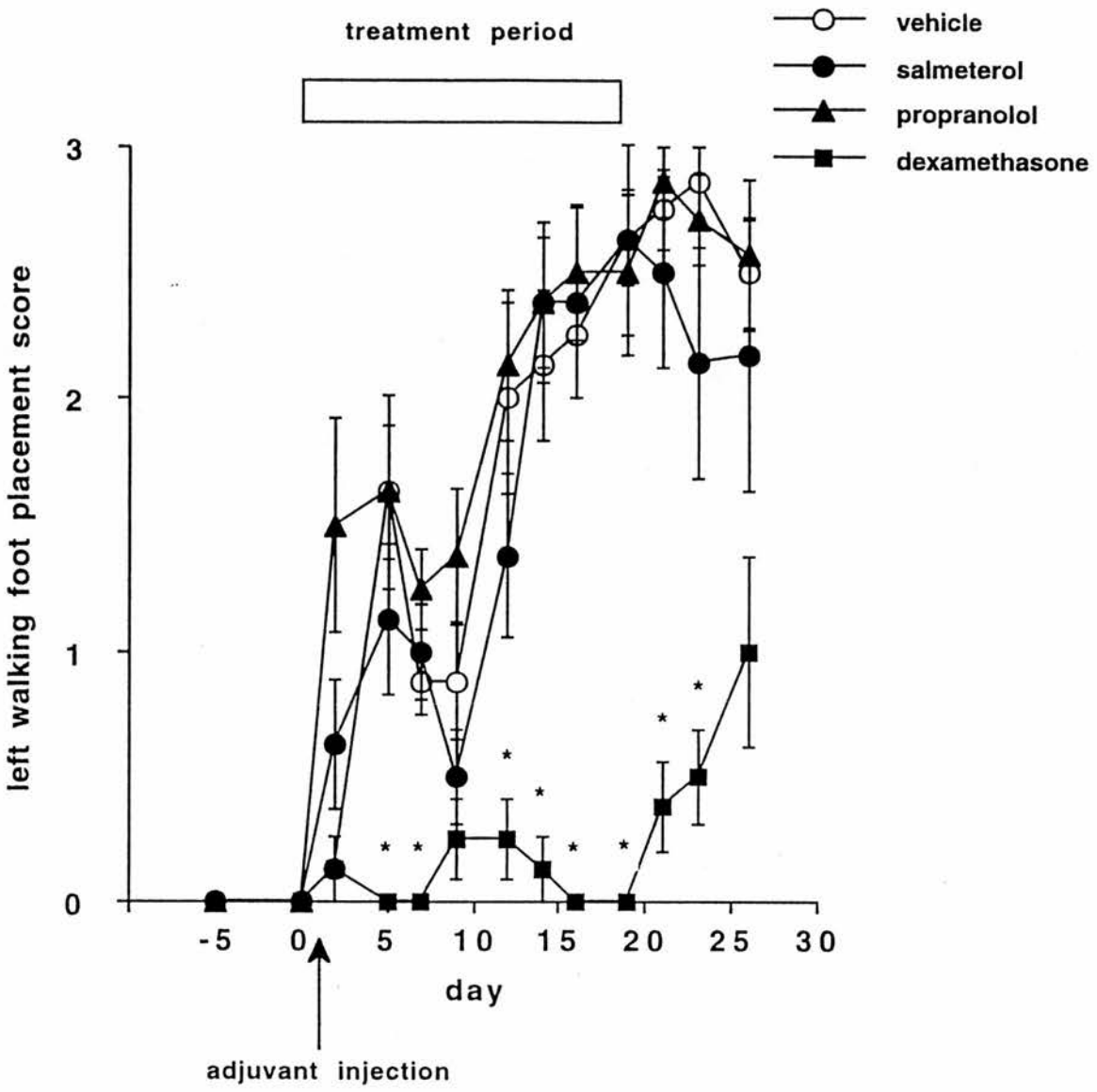



Figure 6.15 Effects of vehicle, salmeterol (50μgkg$^{-1}$, i.p.), propranolol (1mgkg$^{-1}$, i.p.) and dexamethasone (200μgkg$^{-1}$, i.p.) on left walking foot placement scores in rats with adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: * $P<0.05$, Mann Whitney U-test, versus vehicle.

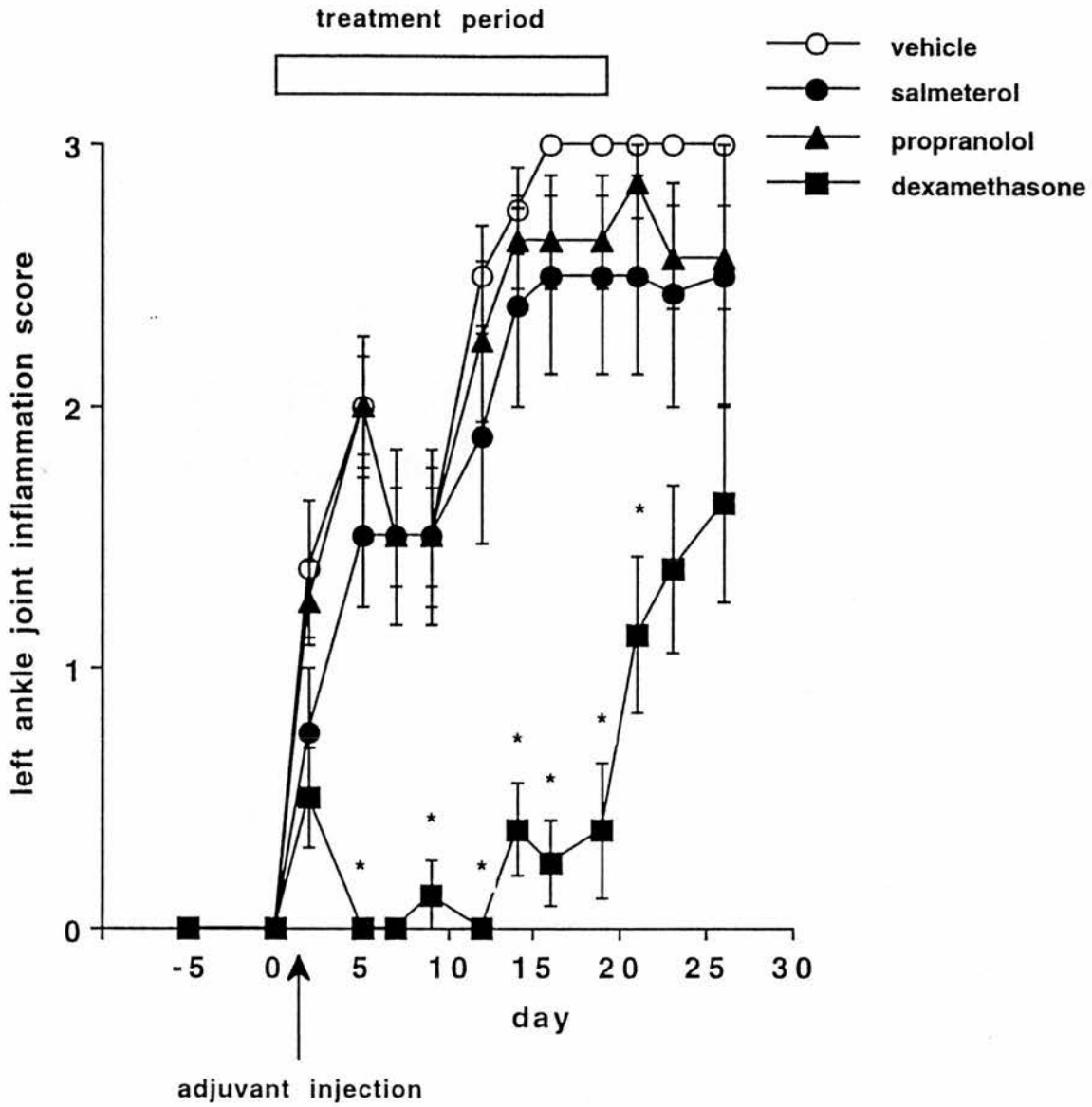



Figure 6.16 Effects of vehicle, salmeterol (50μgkg$^{-1}$, i.p.), propranolol (1mgkg$^{-1}$, i.p.) and dexamethasone (200μgkg$^{-1}$, i.p.) on inflammation scores of adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: * $P<0.05$, Mann Whitney U-test, versus vehicle.

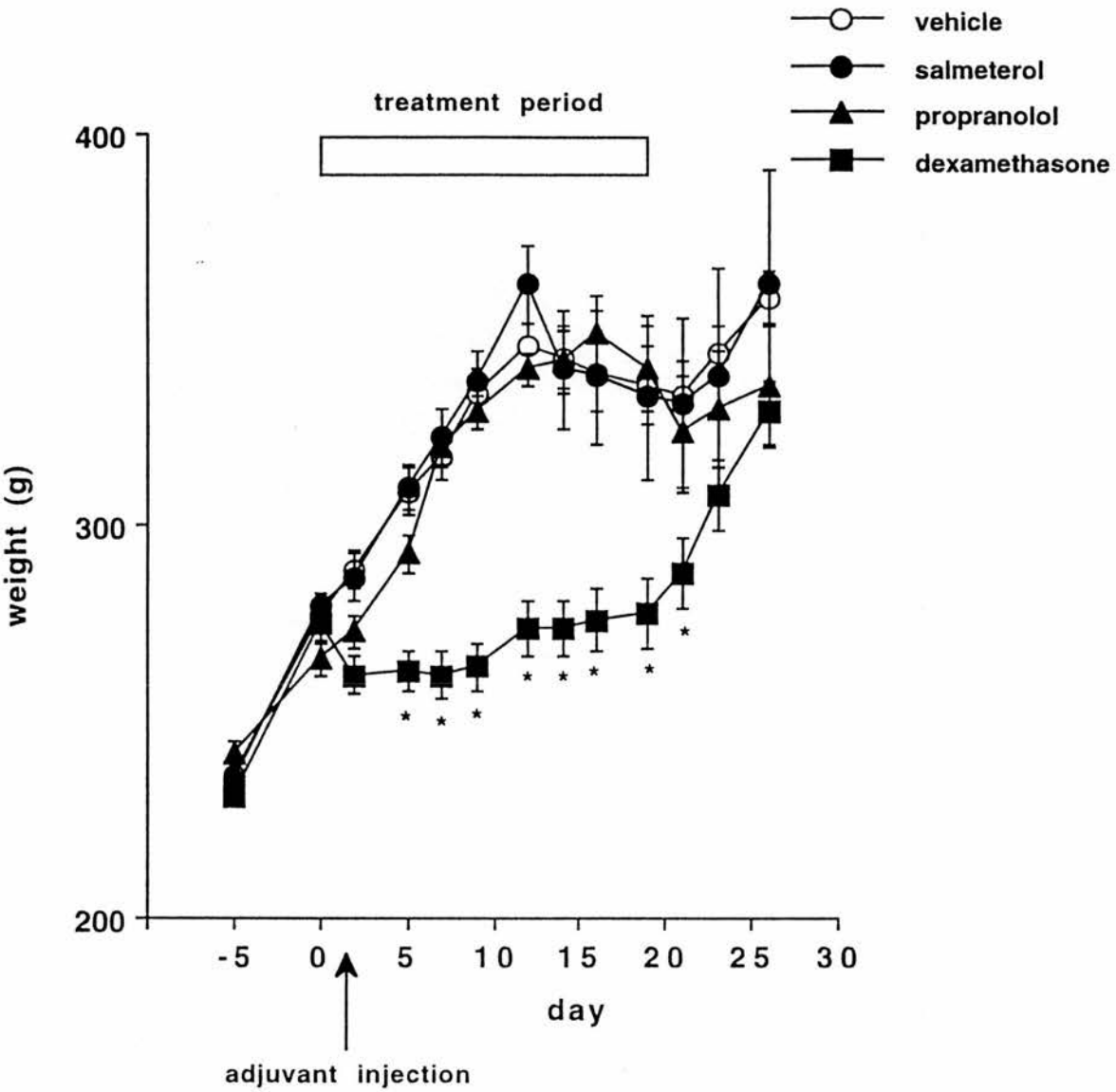



Figure 6.17 Effects of vehicle, salmeterol (50μgkg$^{-1}$, i.p.), propranolol (1mgkg$^{-1}$, i.p.) and dexamethasone (200μgkg$^{-1}$, i.p.) on weight of rats with adjuvant-arthritic left ankle joints. Each point is the mean ± s.e.mean from n=8 experiments. Statistical analysis: $P<0.05$ ANOVA days 2-9 and days 9-19. * $P<0.05$, Mann Whitney U-test, versus vehicle.

terminated, there was an increase in left ankle joint circumference (Figure 6.13), a decrease in left hind limb withdrawal pressure score (Figure 6.14), and increases in left walking foot placement score (Figure 6.15), left ankle joint inflammation score (Figure 6.16) and rat body weight (Figure 6.17).

## 6.4 DISCUSSION

### 6.4.1 $\beta_2$ adrenoceptors and mechanonociceptor discharge from normal and adjuvant-arthritic rats

The results of the present study have shown that neural discharge (spontaneous and mechanically-evoked) from mechanonociceptors in normal rat ankle joints was unaffected by either $\beta_2$-adrenoceptor agonists of either short (salbutamol) or of long (salmeterol) duration, or by the non-selective $\beta$-adrenoceptor antagonist, propranolol. The catecholamines, adrenaline and noradrenaline, also had no effect on neural discharge (spontaneous or mechanically-evoked) from C-afferent fibres in normal ankle joints. These findings are in agreement with the electrophysiological studies in rabbit auricular (Sato & Perl, 1991) and rat skin (Sanjue & Jun, 1989) C-afferent fibres which show the absence of effect of noradrenaline on neural discharge in undamaged tissue. As discussed below, sympathetic efferents can produce pain and hyperalgesia in damaged or diseased tissues. However, in normal tissues, the inability of sympathetic nerve stimulation to produce any change in neural discharge from nociceptors (Roberts & Elardo, 1985; Shea & Perl, 1985; Barasi & Lynn, 1986; Sanjue & Jun, 1989) adds further support to the conclusion that adrenoceptors are not involved in affecting afferent discharges from nociceptors in normal tissues.

In the current investigation, the $\beta_2$-adrenoceptor agonist, salbutamol, produced excitation in only a few afferent units recorded from adjuvant-arthritic ankle joints. This salbutamol-induced excitation was reduced by the $\beta$-adrenoceptor antagonist, propranolol. Even though this salbutamol-induced excitation was small, these results

could suggest that $\beta_2$-adrenoceptors may play a minor role in modulating neural discharges in a small proportion of C-fibre afferents. However, in the present experiments $\beta_2$-adrenoceptor agonists (salbutamol and salmeterol) failed to produce either a change in spontaneous or mechanically-evoked discharge in the vast majority of C-fibre units recorded from adjuvant-arthritic rat ankle joints. Moreover, since $\beta$-adrenoceptor antagonists (non-selective: propranolol; $\beta_2$-selective: ICI118551) had no effect on the enhanced (compared to normal joints) spontaneous discharge and sensitisation of mechanical stimuli associated with chronically arthritic joints, these data suggest that in this model of chronic inflammation (adjuvant-arthritis), $\beta_2$-adrenoceptors are unlikely to play a significant role in the excitation and sensitisation of mechanonociceptors. In accord with this conclusion, an absence of effects of $\beta$-adrenoceptor drugs has been reported in thermal-sensitised cat skin A-$\delta$ nociceptors (Roberts & Elardo, 1985) and in rat myelinated afferents innervating neuromas (Wall & Gutnik, 1974).

### 6.4.2 Effects of adrenaline and noradrenaline on mechanonociceptor discharge

Although adrenaline and noradrenaline had no effect on articular afferent neural discharge (spontaneous and mechanically-evoked) from normal rat ankle joints, they did produce both an increase in spontaneous discharge (excitation) and in the responsiveness to the standard mechanical stimulus (sensitisation) in approximately 50% of articular afferents recorded from chronically inflamed (adjuvant-arthritic) rat ankle joints. In line with these results of the present study, noradrenaline or

adrenaline-induced enhancements in neural discharge have been reported in 78% of rat skin afferents when there was a sustained background discharge induced by a compound algogenic substance (Sanjue & Jun, 1989), and in 42% of rat skin C-fibre (Sato & Perl, 1991), in 40% of cat skin unmyelinated (Häbler et al., 1987) and in most rat myelinated (Wall & Gutnik, 1974; Devor & Jänig, 1981) afferents originating from inflamed tissue (stump neuromata).

In the current study, noradrenaline caused mechanical sensitisation of mechanonociceptors in chronically inflamed (adjuvant-arthritic) joints. In agreement, it has been reported by Gold et al. (1994) that, although noradrenaline alone did not affect mechanical thresholds of rat cutaneous mechanonociceptors, there was a reduction in mechanical thresholds (sensitisation) when noradrenaline was applied in the presence of the calcium ionophore, A23187. Thus, it appears that a local increase in $Ca^{2+}$ concentration accompanying tissue injury (Nayler et al., 1979) is required for noradrenaline-induced mechanical sensitisation.

The delay to the onset of both adrenaline (146 ± 50s) and noradrenaline (124 ± 36s) induced excitation of mechanonociceptors from arthritic joints in the current study were much longer than the delays (10 - 25s) observed by investigators in studies of unmyelinated or myelinated afferents from neuromas (Wall & Gutnik, 1974; Devor & Jänig, 1981 ; Häbler et al., 1987; Koltzenburg & McMahon, 1991; Sato & Perl, 1991). These differences in delays may be explained by differences in the models used (adjuvant-induced arthritis versus neuromas). The delay before the onset of adrenaline and noradrenaline-induced enhancements in neural discharge suggests that the

mechanism of action of these agents may involve the release of other inflammatory mediators such as prostanoids and/or leukotrienes (see discussion below). Similar duration (60 - 120s) of action for adrenaline or noradrenaline-induced excitation were also reported by Sanjue & Jun (1989) and Sato & Perl (1991) in cutaneous C-fibre afferents from neuromas.

Since adrenaline and noradrenaline-induced excitation and sensitisation of mechanonociceptors from arthritic joints were unaffected by propranolol, this indicates that these catecholamines were not acting via β-adrenoceptors to produce their effects. In agreement with these results, it has been shown that activation of afferents ending in a neuroma by adrenaline were unaffected by propranolol (Wall & Gutnick, 1974; Devor & Jänig, 1981). Since adrenaline and noradrenaline are also potent α-adrenoceptor agonists (Bowman & Rand 1980) it is probable that the excitation and sensitisation evoked in the current experiments was due to an action at α-adrenoceptors - whether $\alpha_1$ or $\alpha_2$-adrenoceptors, or a contribution of both, are responsible for the adrenaline and noradrenaline-induced enhancements in neural discharge (excitation and sensitisation) in arthritic joints requires further investigation using selective $\alpha_1$ and $\alpha_2$-adrenoceptor agonists and antagonists. Indeed, electrophysiological recordings from afferent fibres suggest that α-adrenoceptors mediate the actions of adrenaline and noradrenaline, since the α-adrenoceptor antagonist, phentolamine, but not the β-adrenoceptor antagonist, propranolol, blocked the enhancements in neural discharge induced by these catecholamines (Wall & Gutnik, 1974; Devor & Jänig, 1981). Furthermore, the study by Sato & Perl (1991)

points to the involvement of $\alpha_2$-adrenoceptors, since noradrenaline-induced excitation of cutaneous nociceptors following peripheral nerve injury could be blocked by the $\alpha_2$-adrenoceptor antagonists, yohimbine and rauwolscine. However, this study by Sato & Perl (1991) also showed that noradrenaline-induced excitations could be blocked, albeit to a lesser degree, by the $\alpha_1$-adrenoceptor antagonist, prazosin, indicating that there may also be a contribution from $\alpha_1$-adrenoceptors. Regarding the catecholamine-induced mechanical sensitisation of mechanonociceptors observed in the present experiments, $\alpha_2$-adrenoceptors are likely to be involved, since it has been shown that noradrenaline-induced mechanical sensitisation of cutaneous mechanonociceptors was blocked by the $\alpha_2$-adrenoceptor antagonist, yohimbine, but not by the $\alpha_1$-adrenoceptor antagonist, prazosin (Gold et al., 1994).

Since adrenaline and noradrenaline are potent vasoconstrictors, it is possible that the excitation or sensitisation observed with these catecholamines in the present investigation, was due to an effect on blood pressure. However, since $\alpha,\beta$-methylene-ATP (see Section 5) or endothelin (Kelly & McQueen, unpublished) cause large increases in blood pressure without affecting neural discharge (spontaneous or mechanically-evoked) it is unlikely that the increase in blood pressure or local vasoconstriction induced by adrenaline or noradrenaline influenced neural discharge in the current experiments.

#### 6.4.2.1 Location(s) of action of adrenaline and noradrenaline-induced enhancements of discharge from articular mechanonociceptors

Of the various sites at which adrenaline and noradrenaline could cause enhancement of neural discharge (spontaneous and mechanically-evoked) from articular mechanonociceptors in adjuvant-arthritic rat ankle joints, two possibilities include the sympathetic efferent nervous system and the terminals of C-fibre afferents. Regarding the sympathetic efferent nervous system, various studies have suggested that there is an indirect coupling of sympathetic efferents and afferent fibres (see Fitzgerald, 1989; Gonzales et al., 1991; McMahon, 1991; Schaible & Grubb, 1993; Gold et al., 1994). In such a scenario, adrenaline or noradrenaline act on adrenoceptors on sympathetic postganglionic terminals to cause the release of prostaglandins and/or leukotrienes (or other mediators e.g. 5-HT, substance P) which then act on afferent terminals to cause excitation or sensitisation. Indeed the long latency to the onset of adrenaline and noradrenaline-induced excitation observed in the present investigation would propose such an indirect mechanism of action. Further experiments where the effects of adrenaline and noradrenaline are studied in preparations where prostaglandin and/or leukotriene production is blocked would aid in determining whether or not these catecholamines act indirectly. It would be of interest to perform further experiments in order to determine whether sympathetic stimulation or sympathectomy could affect neural discharge from mechanonociceptors in both normal and chronically inflamed (adjuvant-arthritic) joints. Moreover, it would be interesting to determine if chronic inflammation could affect the resting discharge of sympathetic efferents.

Since in the present investigation, adrenaline and noradrenaline affected mechanonociceptor discharge in arthritic joints, but not in normal joints, it would have to be postulated that the sympathetic system undergoes changes under inflammatory conditions, for example, by up-regulation and/or expression of adrenoceptors, or the coupling of adrenoceptors to different second messenger systems in the presence of a raised calcium concentration caused by inflammation (Gold et al., 1994). However, the role of the sympathetic nervous system in affecting afferent neural discharge has been questioned by Scott (1994), who presents powerful arguments that it is more likely to be visceral afferents (terminology also includes peripheral structures), which travel within autonomic nerves, which are the location of action of adrenergic agents. Another possible location of action for adrenaline or noradrenaline-induced increases in afferent discharge in the present study are the afferent nociceptive terminals, where there may be expression or up-regulation of α-adrenoceptors (see Devor, 1991; McMahon, 1991).

### 6.4.3 Behavioural studies of the effects of β-adrenoceptor agonists and antagonists on a localised Freunds adjuvant-induced arthritis in the rat

Two studies were performed to determine the effects of β-adrenoceptor agonists and antagonists in rats with a localised adjuvant-induced arthritis of the left ankle joint. In the first study, the $\beta_2$-adrenoceptor agonist, salmeterol, showed no effects on measures of inflammation (Figures 6.8 & 6.11) or hyperalgesia (Figures 6.9 & 6.10) associated with rats with an established adjuvant-induced arthritis. In the second investigation, treatment (i.p. injections started 2 days before injection of adjuvant)

with the $\beta_2$-adrenoceptor agonist, salmeterol, or the non-selective β-adrenoceptor antagonist, propranolol, was found not to have any effects on the onset and progression (acute and chronic phases) of the localised adjuvant-induced arthritis of the rat ankle joint as determined by parameters of inflammation (Figures 6.13 & 6.16) and hyperalgesia (Figures 6.14 & 6.15). In contrast to these results of the present investigation, it has been shown in the same model that propranolol, or more specifically the $\beta_2$-adrenoceptor antagonists, butoxamine and ICI 118551, attenuated the progression of joint injury when treatment began before or after the onset of experimental arthritis (Levine et al., 1988), and that the $\beta_2$-adrenoceptor agonist, salbutamol, significantly increased joint injury (Coderre et al., 1990; Coderre et al., 1991). There are several explanations which could account for the differences between these observations and those of the present study. For example, in the present study propranolol was injected once daily at a dose of $1 mgkg^{-1}$, whereas a significantly higher dose ($20 mgkg^{-1}$, 3 times daily) was used in the studies by Levine et al. (1988). Since high doses of propranolol can cause effects unrelated to β-adrenoceptor blockade, including membrane stabilisation (Bowman & Rand, 1980), it is possible that propranolol may have caused a reduction in joint severity in the study by Levine et al. (1988) by some non-specific mechanism. Another difference relates to the measurements of adjuvant-arthritis; the studies by Levine et al. (1988) and Coderre et al. (1990; 1991) used radiological scores, whereas the current investigation used other measures (see Section 2.2.1). However, since it has been shown that measurements of limb joint circumference in adjuvant-arthritis correlates well with joint histology (Donaldson et al., 1993), it is unlikely that differences in the

measurements of the adjuvant-induced arthritis between the studies can account for the differences observed to β-adrenoceptor agents.

The failure of salmeterol to enhance experimental arthritis in the current investigation may have been because severity of joint damage was already at a peak. Indeed, it has been reported by Coderre et al. (1991) that salbutamol could not increase joint injury in rats with adjuvant-induced arthritis, although when salbutamol was given to a rat strain (Wistar-Kyoto) which develops only a mild experimental arthritis, there was an exacerbation of the arthritis (Coderre et al., 1991). However, since in the current study, not only did salmeterol treatment not increase the degree of adjuvant-induced arthritis, but neither did it enhance its onset or progression, it appears that salmeterol has no effect on adjuvant-induced arthritis.

$\beta_2$-adrenoceptor agonists such as salbutamol, and in particular longer-duration $\beta_2$-adrenoceptor agonists such as salmeterol (Ball et al., 1991), have been shown to have anti-inflammatory effects including inhibition of mediator (histamine, leukotrienes and prostaglandins) release (Butchers et al., 1979; Butchers et al., 1991), bradykinin-induced plasma protein extravasation (Whelan et al., 1993), vascular permeability and granulocyte accumulation (Whelan & Johnson, 1992) and carrageenan-induced oedema (Green, 1972). In contrast, no anti-inflammatory or analgesic effects were observed in the current investigations using a model of chronic inflammation (adjuvant-induced arthritis). It could be that the rat adjuvant model of arthritis is not capable of detecting slight anti-inflammatory or analgesic actions, even although it can clearly detect the actions of anti-inflammatory drugs such as dexamethasone (Figures

6.8 - 6.17 & Section 6.4.4) or indomethacin (see Section 4), or it could show a difference in the ability of β-adrenoceptor agonists to produce acute and chronic anti-inflammatory effects.

### 6.4.4 Effects of dexamethasone on localised adjuvant-induced arthritis.

The corticosteroid, dexamethasone, has been long established as a drug which significantly reduces inflammation and hyperalgesia in adjuvant-arthritic rats (see Winter & Nuss, 1966; Schmollack & Steup, 1988). Thus, in the present behavioural experiments, involving the study of β-adrenoceptor active drugs, it was decided to inject dexamethasone in one group of rats - this group then functioned as the positive control group. As expected from the literature (see above), dexamethasone in the current investigation was anti-inflammatory and reduced mechanical hyperalgesia. The mechanism of action of dexamethasone involves, following its entry into the cell, interactions with intracellular specific receptors in the nucleus with the subsequent formation of various polypeptides which have anti-inflammatory effects. For example, one such peptide is lipocortin which inhibits phospholipase $A_2$ activity, and thus inhibits the release of arachidonic acid (see Vane & Botting, 1987). Consequently, there is inhibition in the formation of both prostaglandins and leukotrienes. Indeed, in the present studies dexamethasone was more potent at reducing inflammation and hyperalgesia than was the NSAID, indomethacin, which only inhibits the formation of prostaglandins (see Section 4.4.3). This rank order of potency of dexamethasone > indomethacin seen in the current study confirms the same potency order seen by other

investigators in adjuvant-arthritic rats (Winter & Nuss, 1966; Schmollack & Steup, 1988).

## 6.5 SUMMARY

In summary, the results of the present investigation show that β-adrenoceptor agonists and antagonists do not affect neural discharge from mechanonociceptors in either normal or adjuvant-arthritic rat ankle joints - all units recorded were excited by the selective C-fibre excitant, capsaicin. In line with these neuropharmacological studies, the results of complementary behavioural investigations also showed that β-adrenoceptor agonists and antagonists do not affect the inflammation and hyperalgesia associated with chronic arthritis (adjuvant-induced) of the rat ankle joint - the steroid, dexamethasone, was clearly anti-inflammatory and analgesic in this model of arthritis.

Although adrenaline and noradrenaline did not affect neural discharge from mechanonociceptors in normal joints, they did enhance both spontaneous and mechanically-evoked discharge in approximately 50% of C-fibre units recorded from chronically-inflamed (adjuvant-arthritic) joints. Since these effects were not affected by propranolol, this strongly suggests that the catecholamines are not acting at β-adrenoceptors, but rather at α-adrenoceptors. Further studies, which were not possible during the present investigation, would be needed to confirm this.

*SECTION 7*

# *EFFECTS OF BRADYKININ AND ITS ANALOGUES ON ARTICULAR MECHANONOCICEPTORS IN NORMAL AND ARTHRITIC RAT ANKLE JOINTS.*

## 7.1 INTRODUCTION

The levels of plasma kinins are increased markedly during various types of inflammatory reaction, including rheumatoid arthritis in humans (Bhoola et al., 1992; Sharma, 1992), and adjuvant-induced arthritis in animals (VanArman & Nuss, 1969; Reis et al., 1982). Bradykinin (BK) produces many pro-inflammatory effects such as vasodilation, tissue oedema and increased vascular permeability, and is one of the most potent endogenous occurring algogenic agents. In man, BK produces pain when applied to the cantharadin-induced blister base (Keele & Armstrong, 1964; Whalley et al., 1987), or when it is injected intra-arterially (Coffman, 1966), intra-abdominally (Lim et al., 1967), sub-dermally (Ferreira, 1972), intra-muscularly (Jensen et al., 1990a) or intra-dermally (Jensen et al., 1990b). In animal studies, pseudoaffective responses (e.g. vocalisation, dextrorotation, biting, scratching, licking, and reflex increase in blood pressure) are obtained following injection of BK intra-arterially (Guzman et al., 1962; Hashimoto et al., 1964) or intra-articularly (Moncada et al., 1975).

In electrophysiological studies, BK has been shown to excite nociceptors in skin (Beck & Handwerker, 1974; Szolcsányi, 1987; Lang et al., 1990; Dray et al., 1992), viscera (Haupt et al., 1983; Mizumura et al., 1990) and joints (Kanaka et al., 1985). It has also been demonstrated that bradykinin can sensitise the response to mechanical (Mense & Mayer, 1987; Grubb et al., 1991; Birrell et al., 1993) and thermal (Koltzenburg et al., 1992) stimuli.

Evidence that is consistent with a role of BK as a physiological mediator of pain has been provided by autoradiographic studies, where BK receptors were shown to be localised to sensory neurones (Steranka et al., 1988). BK receptors are classified into $B_1$ and $B_2$ subtypes (Regoli & Barabe, 1980). In most models of BK-induced pain, hyperalgesia or inflammation it is the $B_2$ receptor which mediates these actions of BK. Although early generation $B_2$ receptor antagonists (e.g. D-Arg-[Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$]-BK) have been shown to block the hyperalgesic and inflammatory effects of BK, their susceptibility to fast enzymatic degradation (Griesbacher et al., 1989) has limited their therapeutic usefulness. Recently D-Arg-[Hyp$^3$, Thi$^5$, D-Tic$^7$, Oic$^8$]-BK (Hoe140), a subsequent generation, potent, and long-acting $B_2$ receptor antagonist (Bao et al., 1991; Lembeck et al., 1991) has been synthesised and shown to block the actions of BK in various models of acute and chronic inflammation (Wirth et al., 1991; Damas & Remacle-Volon, 1992; Corrêa & Calixto, 1993; Heapy et al., 1993; Griesbacher et al., 1993; Griesbacher et al., 1994).

Evidence has accumulated in recent years which implies that $B_1$ receptors play a role in chronic or persistent pain, hyperalgesia and inflammation. Studies by Farmer et al (1991a) have shown that, although $B_1$ receptors are not present under normal conditions, they could be induced under inflammatory (antigen-induced arthritis) situations. More recently, a role for $B_1$ receptors has been demonstrated in rat models of persistent inflammatory thermal (Perkins & Kelly, 1993) or mechanical (Davis & Perkins, 1994) hyperalgesia. There is also evidence that $B_1$ receptors participate in both phases of the formalin-induced pain model in the mouse (Corrêa & Calixto, 1993).

The aims of the present investigation on articular mechanonociceptors in both normal and adjuvant-arthritic ankle joints were fourfold, namely: 1) to study the actions of BK, 2) to determine whether prostaglandins are involved in the BK-induced responses, 3) to examine the actions of $B_1$-selective receptor agonists and antagonists, and 4) to investigate the effects of the potent $B_2$ receptor antagonist, Hoe140.

## 7.2 MATERIALS AND METHODS

### 7.2.1 Electrophysiological studies

The *in-vivo* preparation, neural recording, off-line analysis and statistical analysis are described in detail in Section 2. In brief, male Wistar rats (normal and adjuvant-arthritic) were anaesthetised with urethane and cannulations performed of the trachea, right carotid artery (blood pressure monitoring) and right femoral artery (retrograde cannulation for close intra-arterial bolus injections of drugs into the left limb). The medial aspect of the left ankle joint was exposed, and nerve fibres were isolated from the PACR nerve. C-fibre afferent discharge (spontaneous and mechanically-evoked) from articular mechanonociceptors was recorded extracellularly, using bipolar platinum-iridium electrodes.

**Protocol**

In normal and arthritic rats log dose-response curves (spontaneous and mechanically-evoked discharge) were constructed to bradykinin (1 - 100μg, i.a.) before and after des-Arg$^9$-Leu$^8$-BK (bradykinin $B_1$ receptor antagonist, 100μgkg$^{-1}$ i.a.), Hoe140 (bradykinin $B_2$ receptor antagonist, 10 - 100μgkg$^{-1}$ i.a.) or indomethacin (cyclo-oxygenase enzyme inhibitor, 10mgkg$^{-1}$ i.a.). In another series of experiments, an attempt was made to construct log dose-response curves (spontaneous and mechanically-evoked discharge) to the $B_1$ receptor agonist, des-Arg$^9$-BK (1 - 100μg, i.a), in normal, and acutely (3 -5 days post-adjuvant) or chronically (19 - 30 days post-adjuvant) arthritic, rats; log dose-response curves to bradykinin (1 - 30μg) were also constructed either before or after injection of des-Arg$^9$-BK (1 - 100μg).

**Data analysis**

In order to compare the effects of BK and des-Arg$^9$-BK on spontaneous discharge, ongoing discharges were quantified as the peak increase in discharge (averaged over 15s) above the control discharge (averaged over 60s prior to drug injection). A significant increase in spontaneous discharge in this analysis was defined as an increase above basal spontaneous discharge of >0.5i.p.s. for units in both normal and arthritic joints. This value was obtained from the results of saline injections in normal (0.41 ± 0.07i.p.s. above basal discharge) and arthritic (0.36 ± 0.11i.p.s. above basal discharge) joints. The delay and duration of the enhancement in spontaneous discharge was also measured when possible.

Since des-Arg$^9$-BK, des-Arg$^9$-Leu$^8$-BK and Hoe140 had no obvious effect on spontaneous discharge, the largest number of impulses both above and below the basal discharge were determined by using an arbitrary time period of 60s over 0 - 15min post-injection of the drug; the control period was defined as the 60s period immediately prior to the addition of the test substance (see Section 2.3.5.2 for details of the formula used). In this analysis a significant change in spontaneous discharge was defined as a change over basal discharge of at least 10 impulses for both normal and arthritic joints. This value of 10 impulses was derived from the results of saline injections in normal (6 ± 2 and 6 ± 3 impulses above and below basal discharge, respectively, 9 units) and arthritic (5 ± 2 and 6 ± 3 impulses above and below basal discharge, respectively, 9 units) joints.

Drug effects on mechanically-evoked discharge were quantified as the peak number of impulses above or below the pre-injection evoked discharge. When there was no obvious effect of the test substance on the response to the standard mechanical stimulus, the peak number of impulses above and below the pre-injection evoked discharge were calculated. A significant change in mechanically-evoked discharge was defined as a change of 2 or more impulses for both normal and arthritic joints over the basal mechanically-evoked discharge. This value of 2 impulses, was derived from the results of saline injections in normal ($1.4 \pm 0.2$ and $1.1 \pm 0.3$ impulses above and below basal discharge, respectively, 8 units) and arthritic ($1.3 \pm 0.3$ and $1.1 \pm 0.2$ impulses above and below basal discharge, respectively, 8 units) joints.

## 7.3 RESULTS

### 7.3.1 *In-vivo* electrophysiology in normal and adjuvant-arthritic rat ankle joints

A total of sixteen normal (twenty one units), four acutely arthritic (five units; 3 -5 days post-adjuvant) and twenty two chronically arthritic (thirty five units; 28 ± 3 days post-adjuvant) joints were examined in this series of experiments. The mean afferent conduction velocities were in the C-fibre range for all the units studied in both normal ($0.72 \pm 0.06$ $ms^{-1}$; range: 0.43 - 1.0 $ms^{-1}$) and arthritic ($0.74 \pm 0.10$ $ms^{-1}$; range: 0.41-1.5 $ms^{-1}$) joints. There was no significant difference in the afferent conduction velocity between normal and arthritic joints ($P>0.05$, Mann-Whitney U-test). Before the addition of any drugs, all the units in both normal and arthritic joints had on-going (spontaneous) neural discharges. However, the spontaneous discharges from arthritic joints (3.9 ± 0.8 i.p.s.: range 1.7 - 11.6 i.p.s.) were significantly ($P<0.05$, Mann-Whitney U-test) greater than those from normal joints (1.1 ± 0.3 i.p.s.: range 0.2 - 1.8 i.p.s.). All the units examined in both normal and arthritic joints were excited by close intra-arterial injection of capsaicin (1-3μg).

### 7.3.2 Effects of bradykinin on spontaneous discharge

#### 7.3.2.1 Bradykinin-induced excitation in normal joints

Twenty one units from fifteen individual experiments were examined for their response to bradykinin (BK). BK(1-30μg)-induced increase in spontaneous afferent discharge was observed in eighteen units (86%) from thirteen experiments (see

Figure 7.1 for a typical BK-induced excitation). The remaining three units from two experiments were not affected by BK (1-100μg) although they were excited by capsaicin (1μg, i.a.).

Examination of dose-dependency was carried out on eleven responsive units from eight experiments. BK(1 - 30μg)-induced dose-dependent excitatory responses were observed in nine units from seven joints (Figure 7.2). In the remaining two units (one experiment) desensitisation occurred at the highest dose of BK (30μg).

The delay to the onset of BK-induced excitation was similar (approximately 60s) for the two lower doses of BK (1 and 3μg) but was more rapid (approximately 10s) for the higher doses (10 and 30μg) of BK (Figure 7.3). The duration of the BK-induced excitation was similar (approximately 100s) for lower doses of BK but was longer (>150s) for higher ones (Figure 7.4).

#### 7.3.2.2 Bradykinin-induced excitation in arthritic joints

The effects of BK were determined on thirty five units from twenty two experiments. Thirty one units (89%) from nineteen experiments showed an increase in spontaneous discharge following the application of BK (1-100μg); Figure 7.5 shows a typical BK-induced excitation response. In the remaining four units (three experiments) no excitatory responses were obtained to BK (1 - 100μg) although these were responsive to capsaicin (1 - 3μg, i.a.).

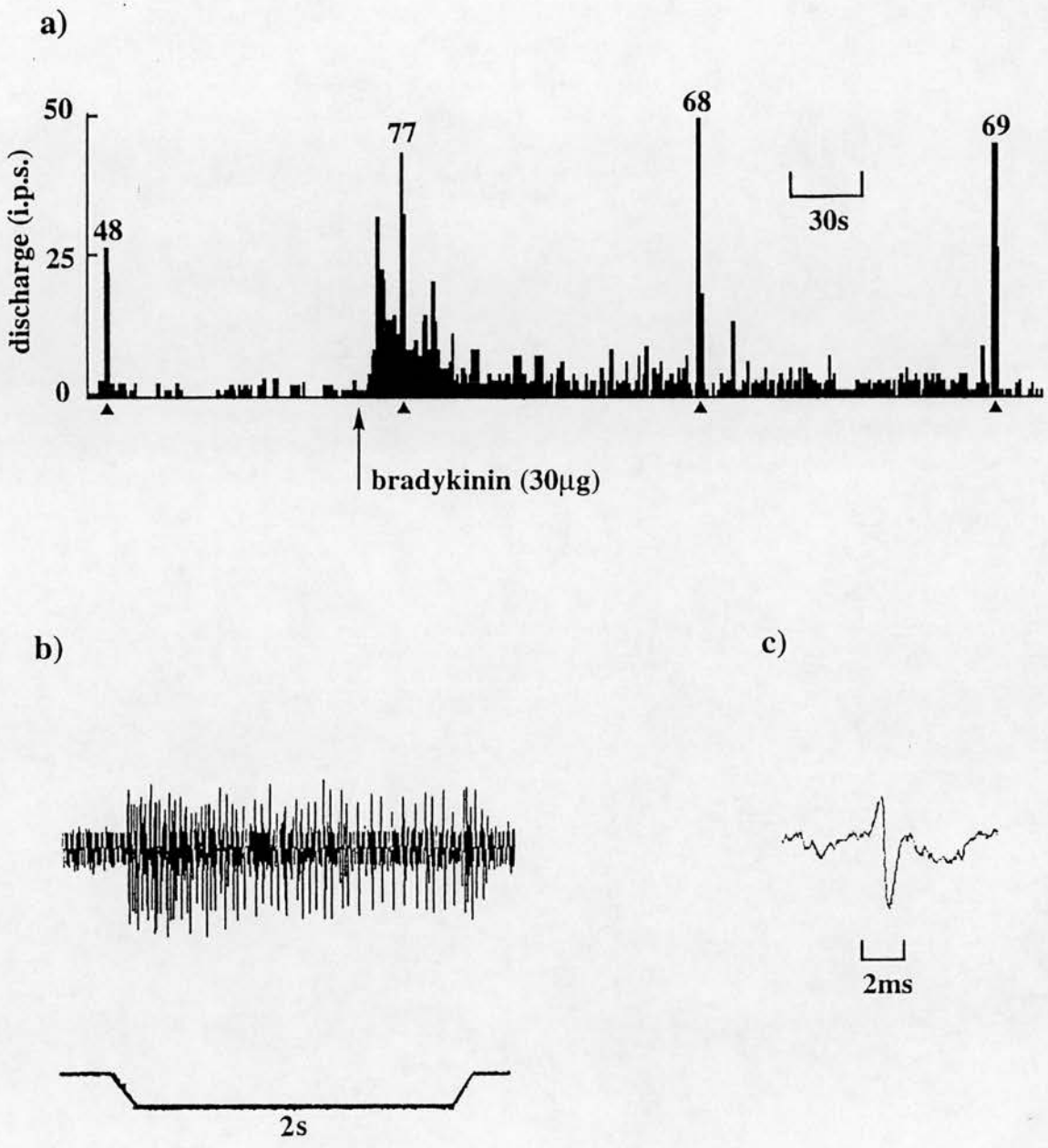



Figure 7.1 (a) Computer generated plot illustrating the excitatory and sensitising effects of bradykinin (30μg, i.a.) on a single C-fibre (conduction velocity 0.6ms$^{-1}$) unit from a normal rat ankle joint. Each bar represents a 1s time interval. Arrowheads indicate the application of the mechanical stimulus. The number of impulses evoked by the mechanical stimulus is given above each response. (b) Neurogram showing response of the unit to a mechanical indentation stimulus (waveform below neurogram). (c) A fast oscilloscope sweep of the unit that was counted.

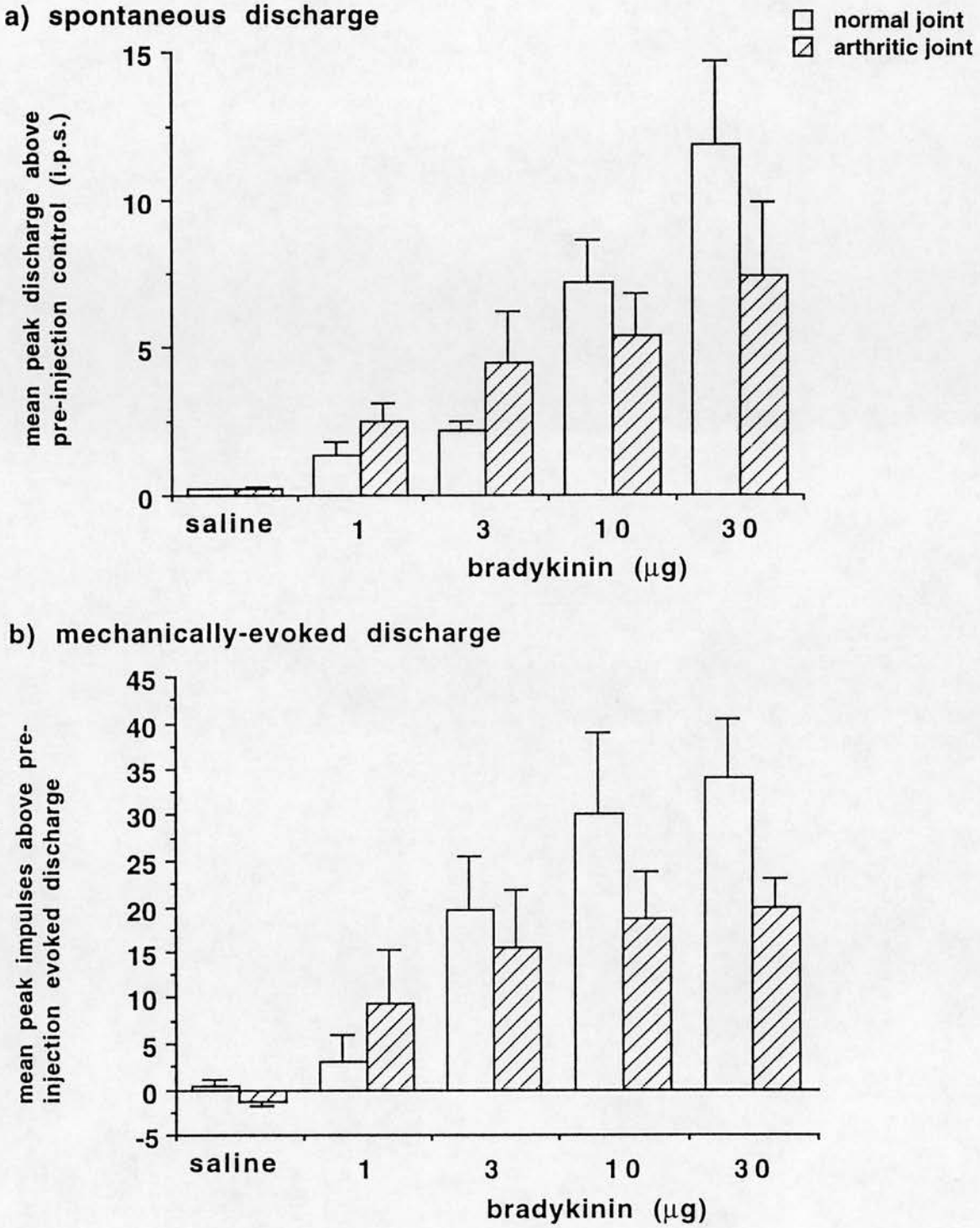



Figure 7.2 Comparison between normal and arthritic (25 ± 5 days post-adjuvant) joints of the effects of bradykinin (1-30μg, i.a.) on (a) spontaneous and (b) mechanically-evoked discharge. Each point is the mean ± s.e.mean from 8 - 10 units (6 - 10 individual experiments). The mean ± s.e.m. pre-injection spontaneous discharge before the injection of any drugs in units recorded from normal and arthritic joints was 1.25 ± 0.12 and 3.89 ± 1.03, respectively. The mean ± s.e.m. pre-injection mechanically-evoked discharge before the injection of any drugs in units recorded from normal and arthritic joints was 40 ± 13 and 47 ± 9, respectively.

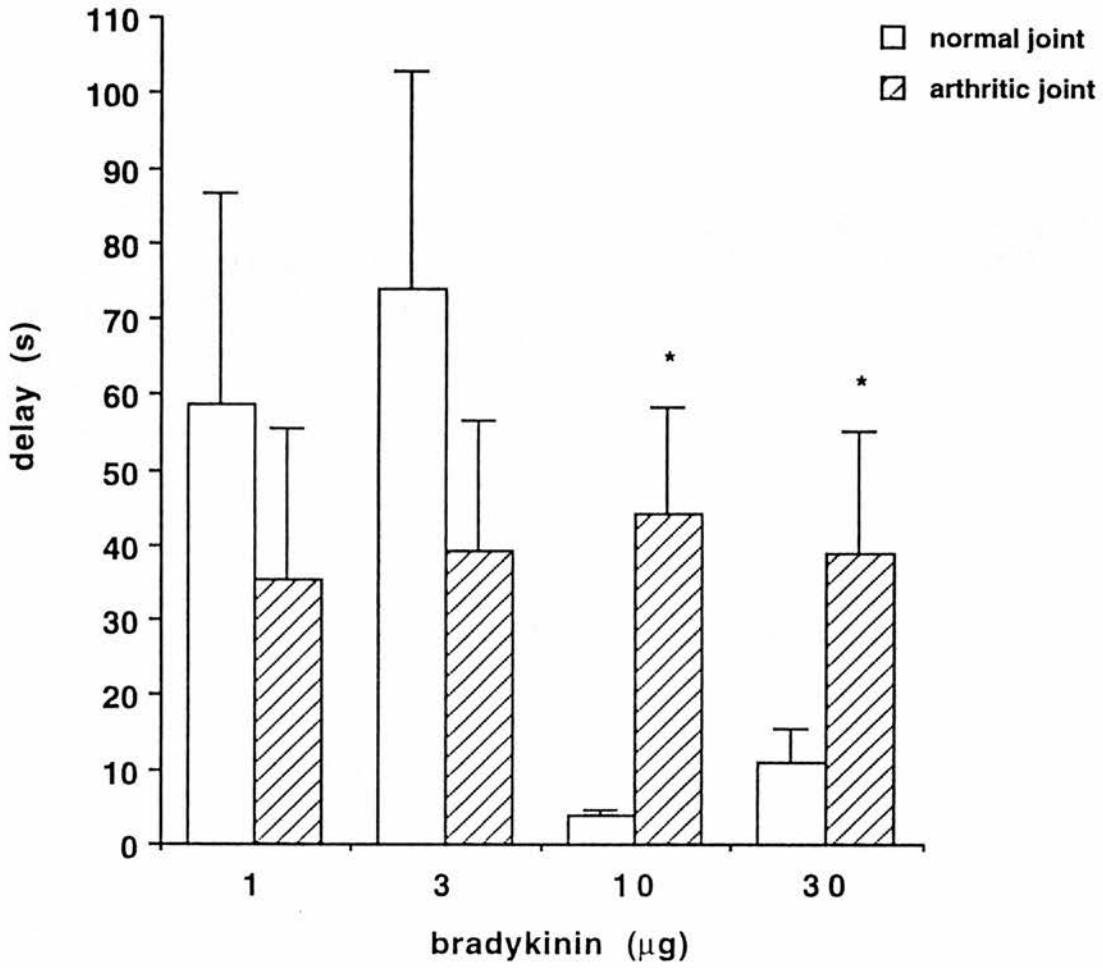



Figure 7.3 Comparison between normal and arthritic joints of the delay to the onset of bradykinin(1-30μg)-induced increase in spontaneous discharge. Each point is the mean ± s.e.mean from 9 - 11 units (7 - 10 individual experiments).* P<0.05 Mann Whitney U-test, versus normal joints.

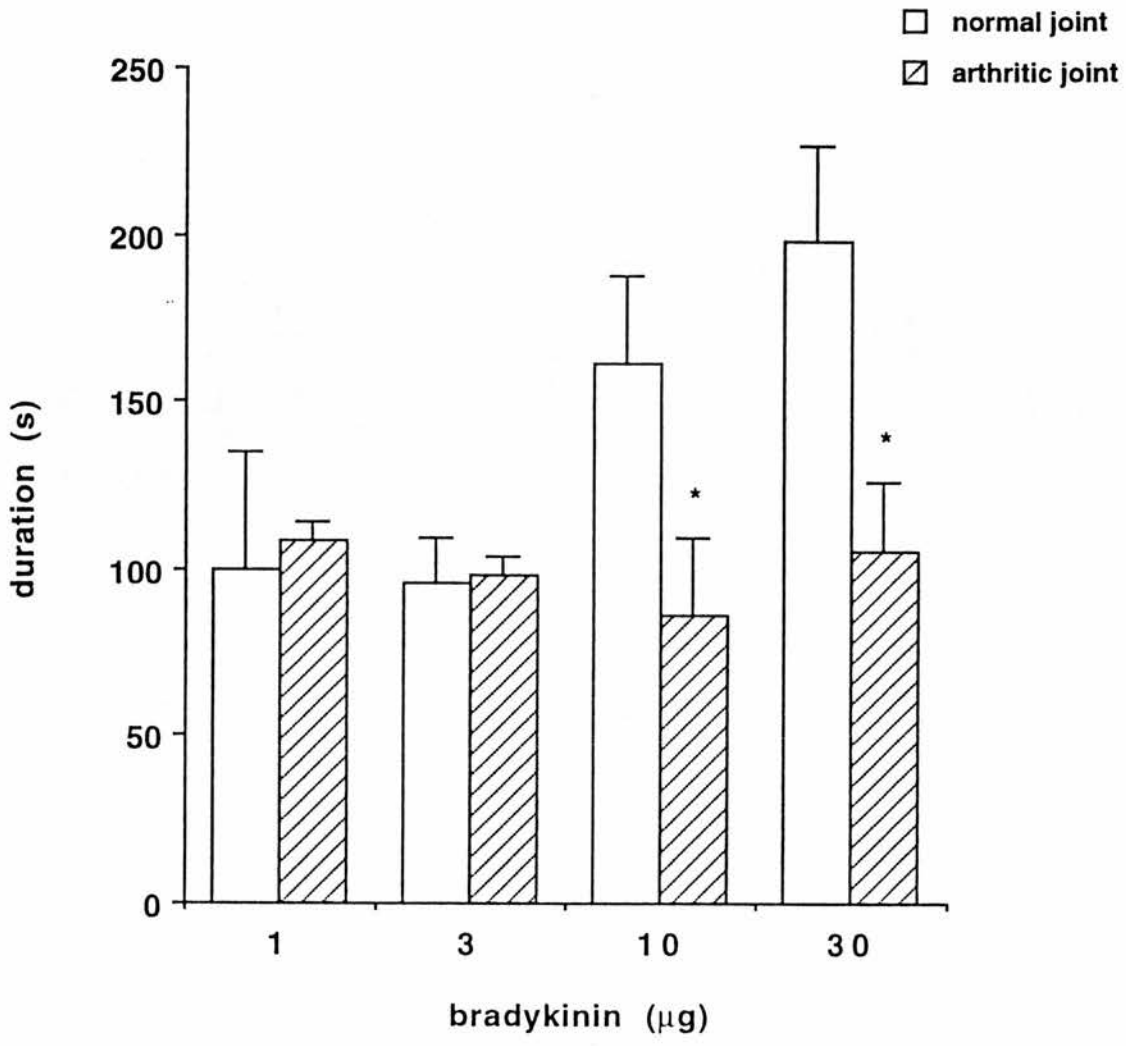



Figure 7.4 Comparison between normal and arthritic joints of the duration of bradykinin(1-30μg)-induced increase in spontaneous discharge. Each point is the mean ± s.e.mean from 9 - 11 units (7 - 10 individual experiments).* $P<0.05$ Mann Whitney U-test, versus normal joints.

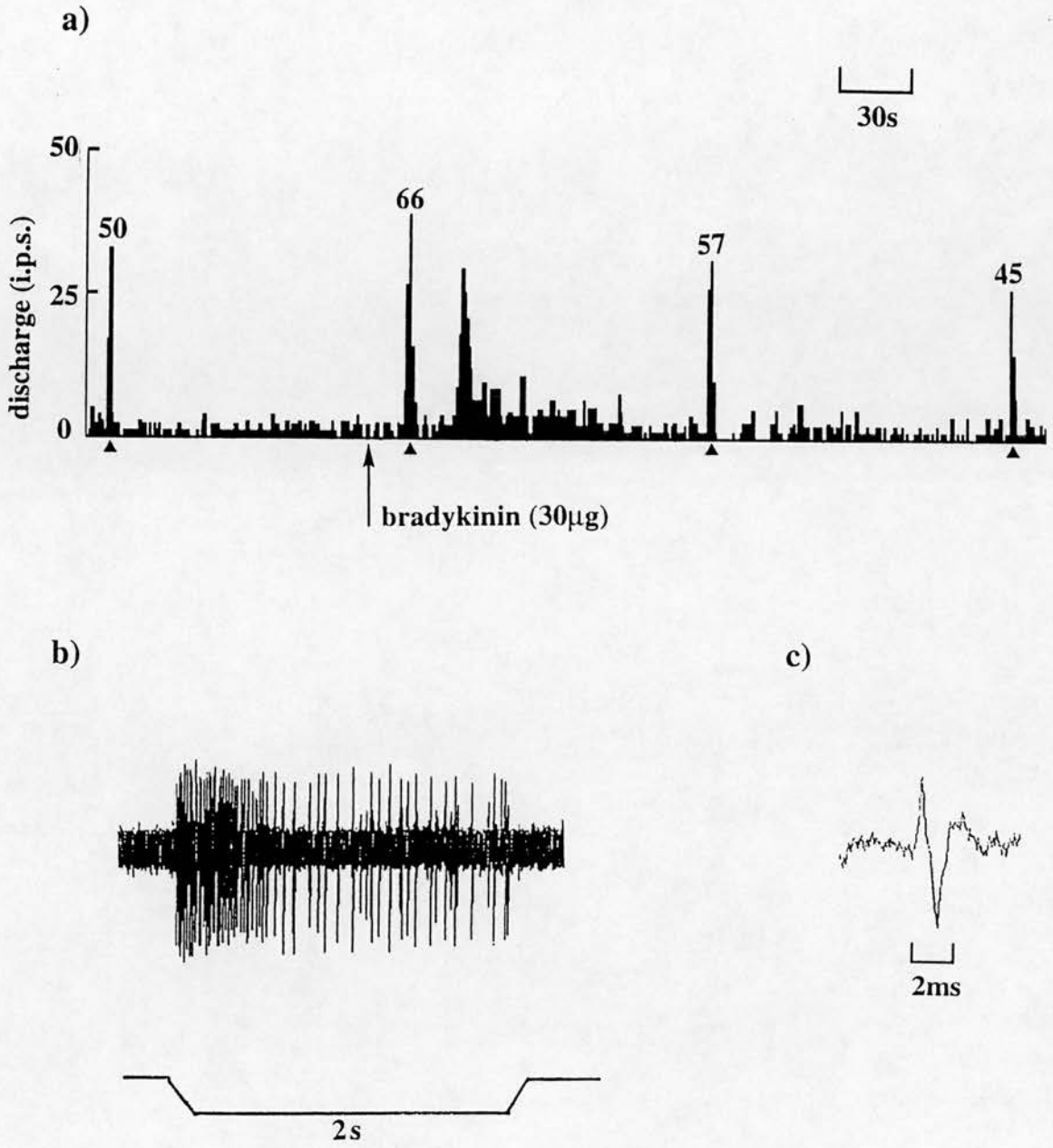



Figure 7.5 (a) Computer generated plot illustrating the excitatory and sensitising effects of bradykinin (30μg, i.a.) on a single C-fibre (conduction velocity 0.6ms$^{-1}$) unit from an adjuvant-arthritic rat ankle joint. Each bar represents a 1s time interval. Arrowheads indicate the application of the mechanical stimulus. The number of impulses evoked by the mechanical stimulus is given above each response. (b) Neurogram showing response of the unit to a mechanical indentation stimulus (waveform below neurogram). (c) A fast oscilloscope sweep of the unit that was counted.

Investigation of dose-dependency was carried out on fifteen responsive units from twelve arthritic (25 ± 5 days post-adjuvant) joints. BK(1 - 30μg)-induced dose-dependent excitation was observed in eleven units from ten arthritic joints (Figure 7.2). In the remaining four units (two experiments) desensitisation occurred at higher doses of BK (10 - 30μg).

BK(10 and 30μg)-induced excitation was greater in normal joints than in arthritic joints, although this difference did not reach statistical significance ($P>0.05$, Mann Whitney U-test)(Figure 7.2).

The delay to the onset of the BK-induced excitation in arthritic joints was similar across the dose (1 - 30μg) range tested (Figure 7.3). In comparison with units in normal joints, the units recorded in arthritic joints showed significantly longer delays to the onset of the BK (10 and 30μg)-induced increase in spontaneous discharge (Figure 7.3).

The durations of the BK-induced excitation were similar (approximately 100s) across the dose (1 - 30μg) range tested (Figure 7.4). The duration of BK (10 and 30μg)-induced excitation in units recorded from arthritic joints was significantly lower than the corresponding duration obtained in normal joints (Figure 7.4).

### 7.3.3 Effects of bradykinin on the responsiveness to mechanically-evoked stimuli

#### 7.3.3.1 Bradykinin-induced sensitisation of mechanically-evoked discharge in normal joints

Twenty units from sixteen experiments were examined for their response to BK. Of these, sixteen units (80%) from thirteen experiments showed enhancements in the responsiveness to the application of the standard mechanical stimulus following the application of BK (1-100μg); Figure 7.1 for a typical BK-evoked sensitisation. In the remaining four units (20%) from three normal joints there were no significant effects of BK on mechanically-evoked discharge.

Eleven units from nine normal joints were examined for the dose-dependency of their response to BK (1 - 30μg). Dose-dependent sensitisations to mechanical stimuli were obtained in eight units from six experiments (Figure 7.2). In the remaining three units (three experiments) there was no dose-dependent sensitisation.

#### 7.3.3.2 Bradykinin-induced sensitisation of mechanically-evoked discharge in arthritic joints

Sixteen units (fifteen experiments) were examined for their response to BK. Fourteen units (88%) from thirteen experiments showed that BK(1-100μg) increased responsiveness to the applied standard mechanical stimulus; Figure 7.5 for a typical BK-induced sensitisation. In the remaining two units, from two arthritic joints, BK had no effect on mechanically-evoked discharge.

The dose-dependency of BK (1 - 30μg) in enhancing mechanically-evoked discharge was investigated on thirteen responsive units from twelve arthritic joints (25 ± 5 days post-adjuvant). In eleven units (ten experiments) BK (1 - 30μg) caused dose-dependent enhancements in the response to the mechanical stimulus (Figure 7.2). In the remaining two units (two experiments) there was no dose-dependent increase in the response to mechanical stimuli.

Although no statistical difference ($P>0.05$, Mann-Whitney U-test) was found for BK-induced sensitisation between normal and arthritic joints, there was a trend for BK-induced sensitisation (3-30μg) to be of greater magnitude in normal joints than in arthritic joints (Figure 7.2).

#### 7.3.3.3 Delay and duration of bradykinin-induced sensitisation in normal and arthritic joints

Since the mechanical stimulus was applied every 2min it was not possible to obtain a precise measurement of latency or duration of BK-induced enhancements of mechanically-evoked discharge. Nevertheless, responses to mechanical stimuli in either normal or arthritic ankle joints were increased (sensitised) at the first, second, or third mechanical stimulus after the injection of BK. The duration of the BK-induced sensitisation lasted typically between one and five mechanically-evoked stimuli. There appeared to be no correlation between the dose of BK and the delay or duration of the BK-induced sensitisation.

### 7.3.4 Reproducibility of bradykinin-induced responses in normal and arthritic joints

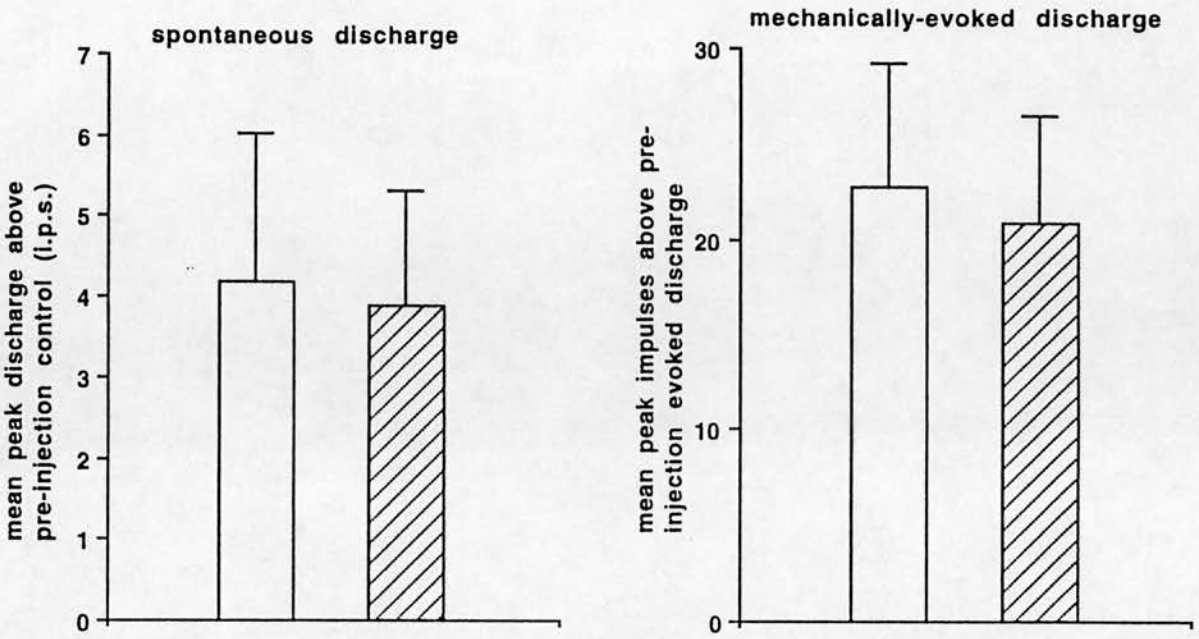
The excitation and sensitisation induced by BK (10μg) were not significantly changed upon the subsequent application (10 - 20min after first injection) of BK (10μg) in units recorded from either normal or arthritic ankle joints (Figure 7.6).

### 7.3.5 Correlations between bradykinin-induced excitation and sensitisation

Although most units from normal and arthritic joints were both excited and sensitised by BK, this was not always found to be the case. Thus, three units in normal joints which showed BK-induced dose-dependent increases in the responsiveness to mechanical stimuli were susceptible to a reduced BK-induced excitation at higher doses of BK (10 - 30μg), and four units which showed no sensitisation following BK, were excited by BK in a dose-dependent manner. Similarly, in arthritic joints, two units which showed BK-induced dose-dependent sensitisation to mechanical stimuli were susceptible to reduced BK-evoked excitation at higher doses of BK (10 - 30μg). Moreover, three units which showed no change in the responsiveness to mechanical stimuli following injection of BK were excited by BK in a dose-dependent manner.

There was no apparent correlation between the durations of BK-induced excitation and sensitisation, as it was possible to have BK-induced sensitisation outlasting the BK-induced excitation or vice-versa (Figure 7.7).

a) normal joint




b) arthritic joint

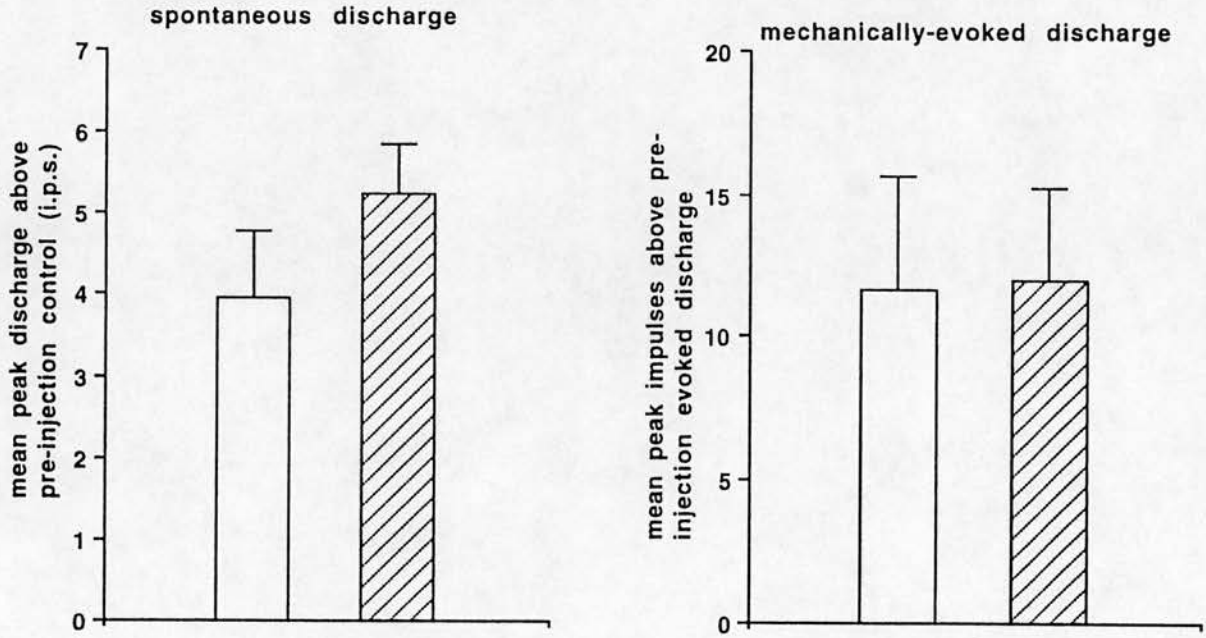



Figure 7.6 Reproducibility of bradykinin (10μg, i.a.)-induced enhancement of spontaneous and mechanically-evoked discharge in (a) normal and (b) arthritic joints. Open bars represent the first injection of bradykinin and hatched bars the second (administered 10 - 20min after the first injection). Each point is the mean ± s.e.mean recorded from 6units (6 individual experiments). There was no difference in the response between the first and second injection of bradykinin ($P>0.05$, Wilcoxon).

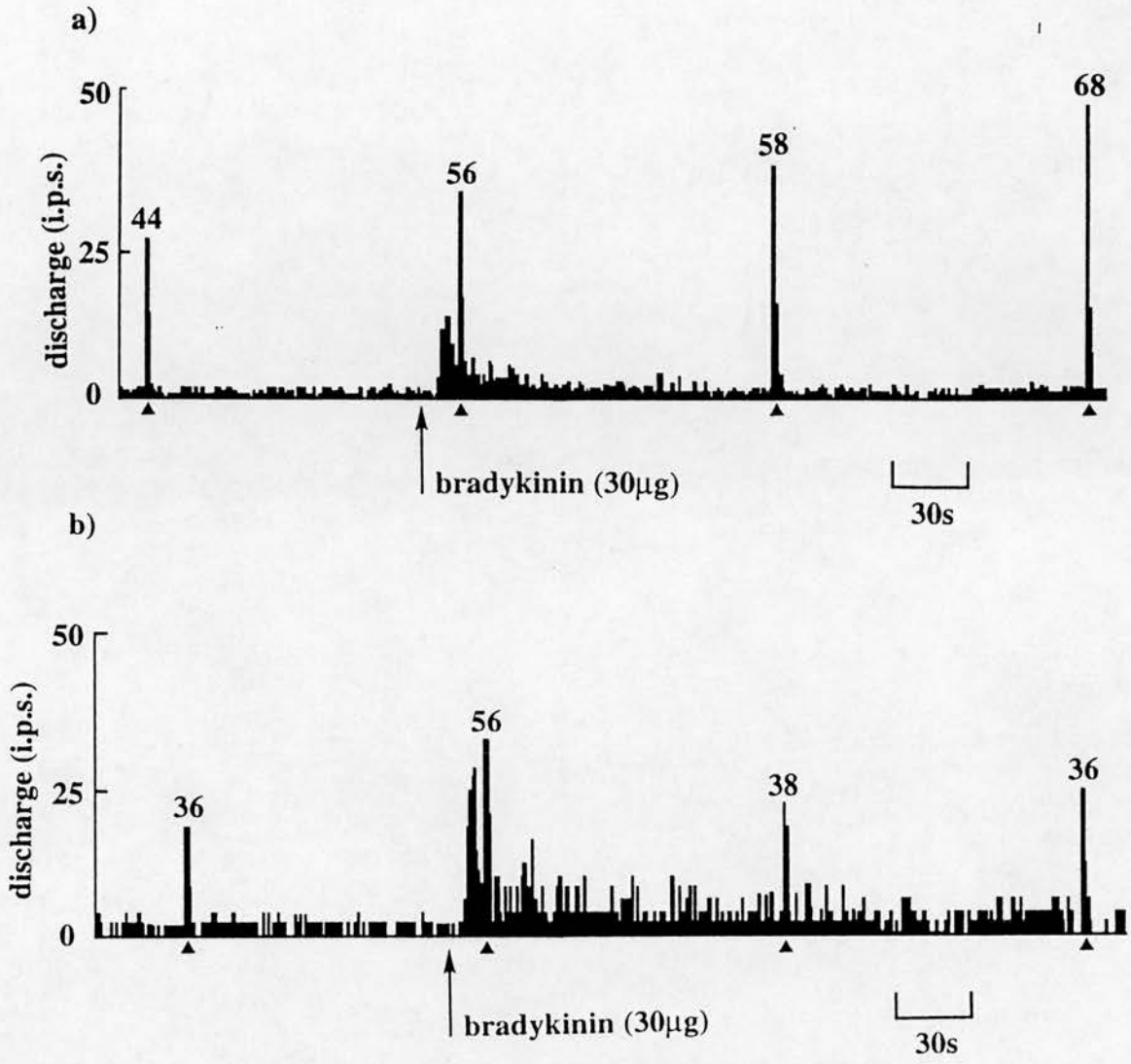



Figure 7.7 Computer generated plots illustrating (a) the longer response duration of bradykinin-induced enhancement in mechanically-evoked discharge as compared to the bradykinin-induced increase in spontaneous discharge and (b) the longer duration of bradykinin-induced enhancement in spontaneous discharge as compared to the bradykinin-induced enhancement in mechanically-evoked discharge. The plots in (a) and (b) were obtained from units recorded from different normal joints; conduction velocity of unit recorded in (a) $0.74ms^{-1}$ and in (b) $0.89ms^{-1}$. Plots illustrating similar differences in the duration of bradykinin-induced responses were also observed in arthritic joints (data not shown). Each bar represents a 1s time interval. Arrowheads indicate the application of the mechanical stimulus. The number of impulses evoked by the mechanical stimulus is given above each response.

### 7.3.6 Effects of indomethacin on bradykinin-induced responses in normal and arthritic joints

Indomethacin (10mgkg$^{-1}$, i.a.) decreased spontaneous and mechanically-evoked discharge (see Section 4) but did not have any significant effect on the BK(1-30μg)-induced increase in spontaneous or mechanically-evoked discharge in units recorded from either normal (Figure 7.8) or arthritic (Figure 7.9) joints ($P>0.05$, Wilcoxon).

### 7.3.7 Bradykinin $B_1$ receptor studies on articular mechanonociceptors

#### 7.3.7.1 $B_1$ receptor studies in normal joints

The $B_1$ receptor agonist, des-Arg$^9$-BK (1-100μg), had no effect on either spontaneous (delta x analysis, see Section 2.3.5.2) or mechanically-evoked discharge in any of the units recorded in normal joints (Figure 7.10). In another analysis, delta $\Sigma\bar{x}$ (see Section 2.3.5.2), des-Arg$^9$-BK (1-100μg) still had no significant effect on spontaneous discharge ($P>0.05$, Mann Whitney U-test, versus saline). All units recorded in this series of experiments were responsive to BK (1 - 10μg, i.a.). The $B_1$ receptor antagonist, des-Arg$^9$-Leu$^8$-BK (1mgkg$^{-1}$, i.a.), did not affect either the BK(1-30μg)-induced excitation nor the BK-evoked enhancement of the responsiveness to the standard mechanical stimulus in any of the units tested (Figure 7.11).

#### 7.3.7.2 $B_1$ receptor studies in acute and chronic arthritic joints

des-Arg$^9$-BK (1-100μg), did not alter spontaneous (delta $\bar{x}$ analysis, see Section 2.3.5.2) or mechanically-evoked discharge in any of the units recorded from acutely-

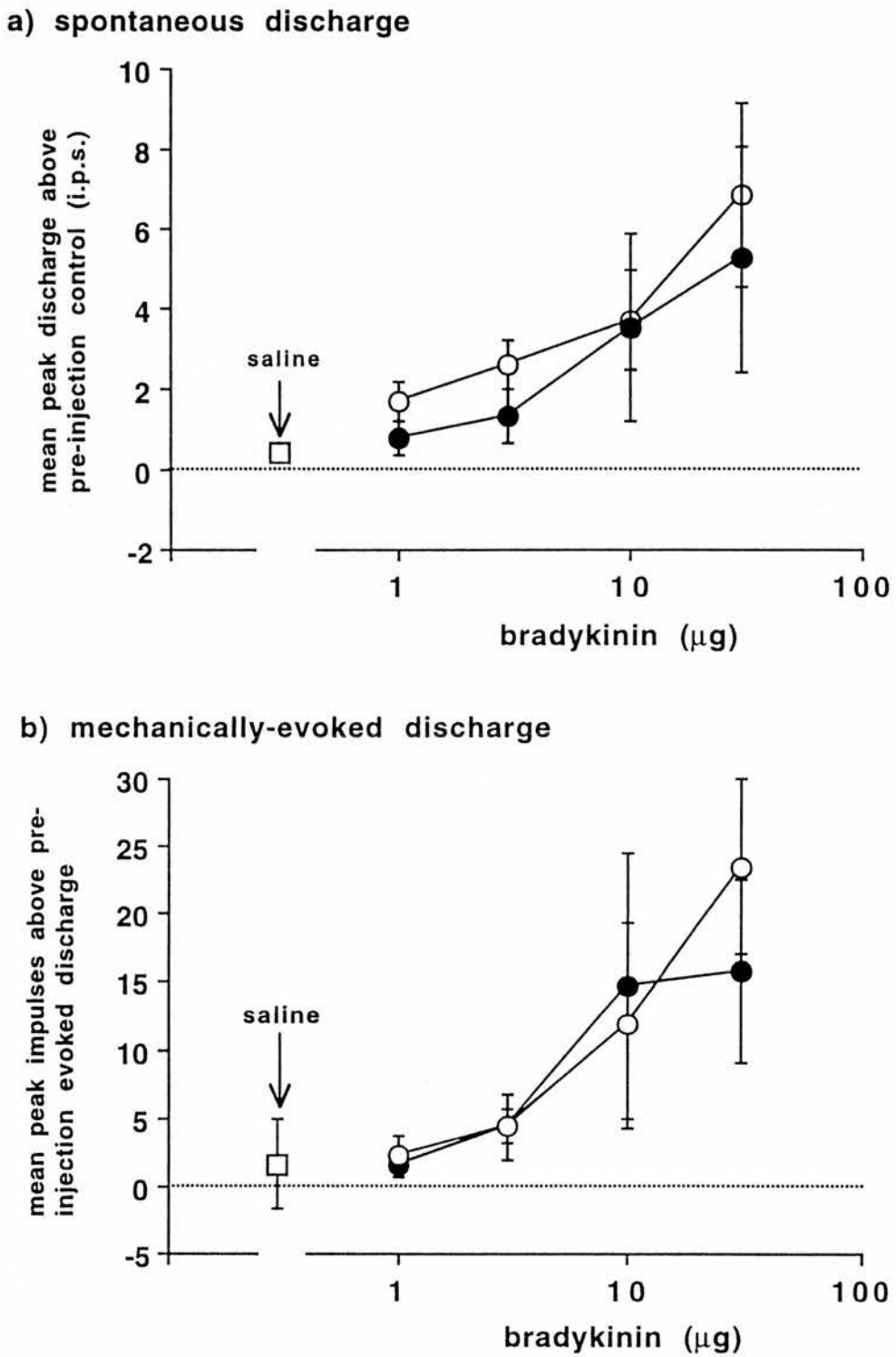



Figure 7.8 Bradykinin-induced enhancement of (a) spontaneous and (b) mechanically-evoked discharge before ( ○ ) and after ( ● ) indomethacin (10mgkg$^{-1}$, i.a.) in normal joints. Each point is the mean ± s.e.mean from 5 -7 units (5 - 6 individual experiments).

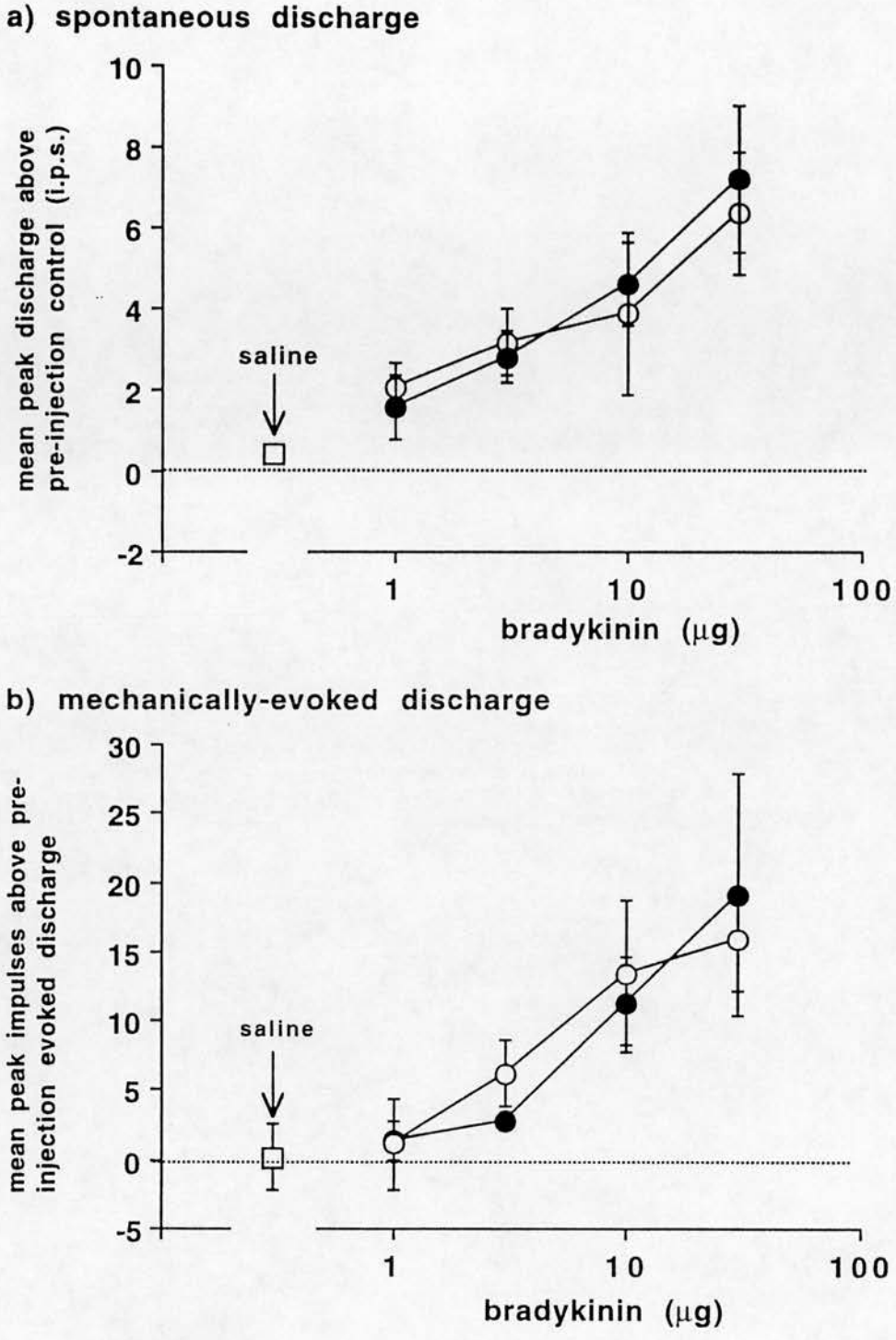



Figure 7.9 Bradykinin-induced enhancement of (a) spontaneous and (b) mechanically-evoked discharge before ( ○ ) and after ( ● ) indomethacin (10mgkg$^{-1}$, i.a.) in chronically arthritic joints (21-27 days post-adjuvant). Each point is the mean ± s.e.mean from 7 - 10 units (5 - 6 individual experiments).

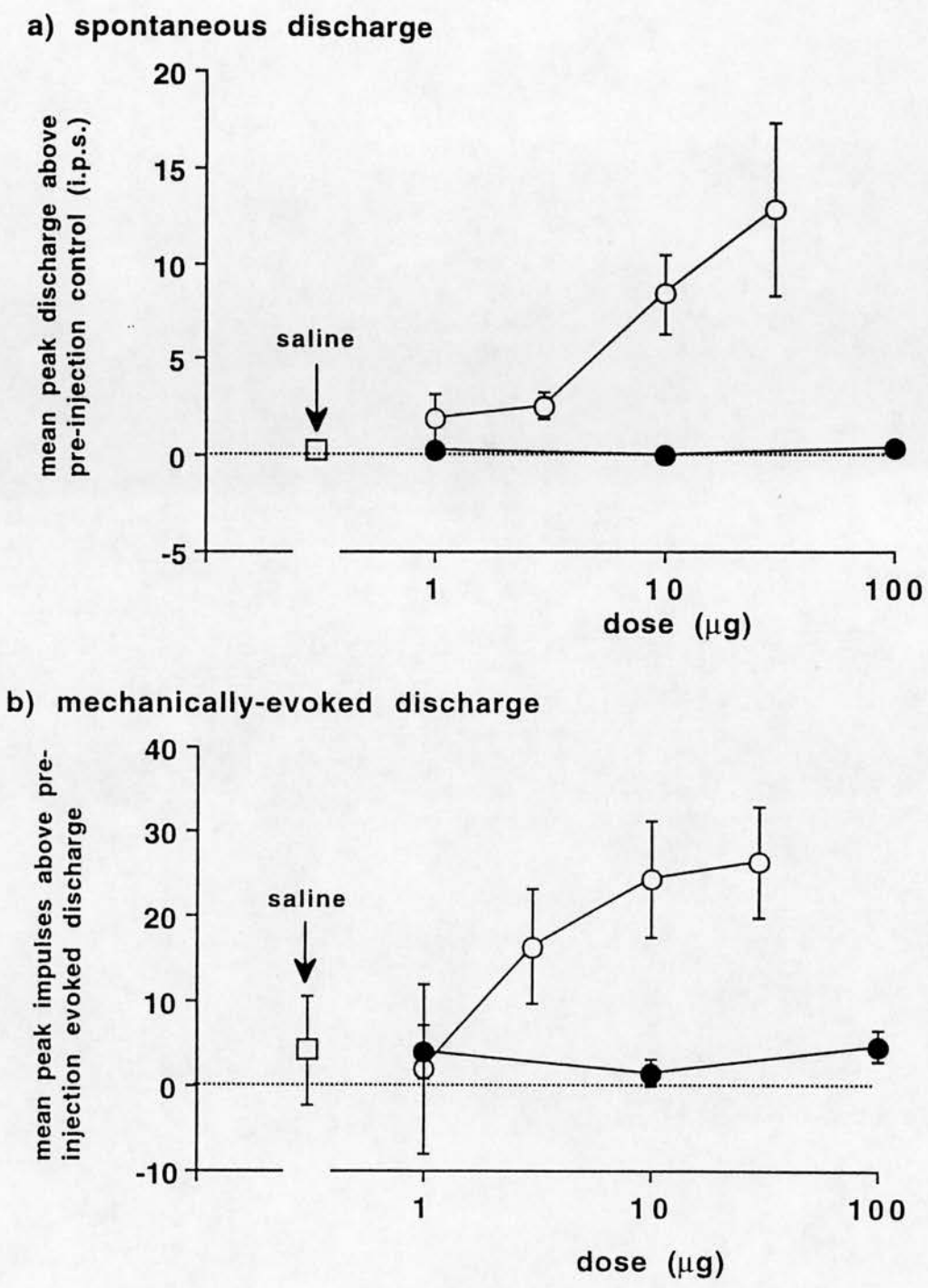



Figure 7.10 Comparison of bradykinin ( ○ ) and des-Arg$^9$-BK ( ● ) on (a) spontaneous and (b) mechanically-evoked discharge from normal joints. Each point is the mean ± s.e.mean from 4 - 5 units (3 - 4 individual experiments).

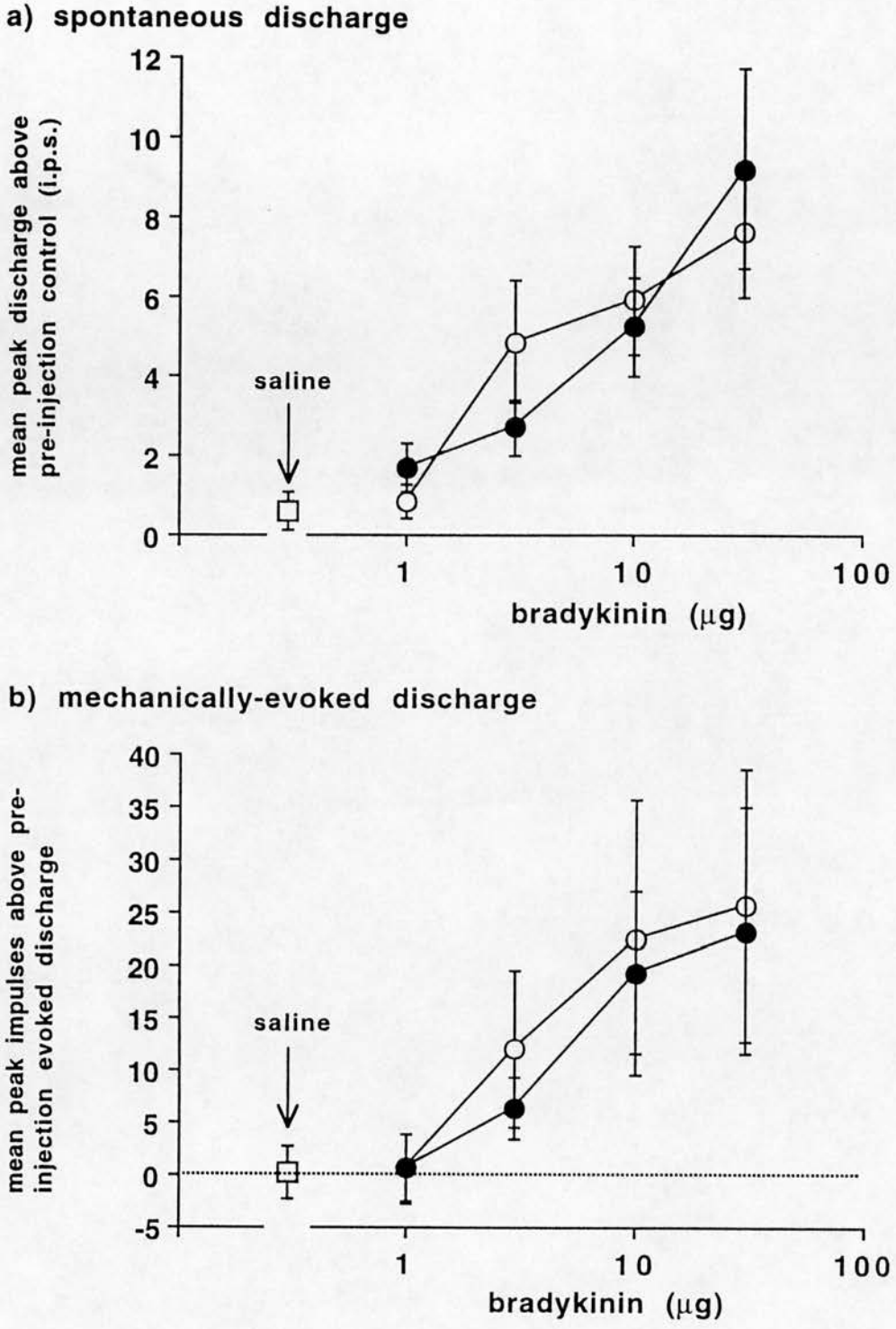



Figure 7.11 Bradykinin-induced enhancement of (a) spontaneous and (b) mechanically-evoked discharge before ( ○ ) and after ( ● ) des-Arg$^{9}$-Leu$^{8}$-BK (1mgkg$^{-1}$, i.a.) in normal joints. Each point is the mean ± s.e.mean from 3 - 4 units (3 - 4 individual experiments).

(3-5 days post adjuvant, Figure 7.12) or chronically-(19-30 days post-adjuvant, Figure 7.13) inflamed joints. In another analysis, delta $\Sigma x$ (see Section 2.3.5.2), des-Arg$^9$-BK (1-100μg) still had no significant effect on spontaneous discharge ($P>0.05$, Mann Whitney U-test, versus saline). All units recorded in this series of experiments were responsive to BK (1 - 10μg, i.a.). BK(1-30μg)-induced enhancements in spontaneous and mechanically-evoked discharge were unaffected in the presence of des-Arg$^9$-Leu$^8$-BK (1mgkg$^{-1}$, i.a.) in any of the units recorded from arthritic joints at either 3-5 days (Figure 7.14) or 15-26 days (Figure 7.15) post-injection of adjuvant.

#### 7.3.7.3 Lack of effects of des-Arg$^9$-Leu$^8$-bradykinin on spontaneous and mechanically-evoked discharge in normal and arthritic joints.

In units recorded from normal joints, des-Arg$^9$-Leu$^8$-BK (1mgkg$^{-1}$, i.a.) had no significant effect on either spontaneous (Table 7.1) or mechanically-evoked (Table 7.2) discharge. Similarly, des-Arg$^9$-Leu$^8$-BK (1mgkg$^{-1}$, i.a.) had no significant effect on either spontaneous (Table 7.3) or mechanically-evoked (Table 7.4) discharge recorded in arthritic joints.

### 7.3.8 Effects of Hoe140 on bradykinin-induced responses

#### 7.3.8.1 Effects of Hoe140 on bradykinin-induced responses in normal joints

In units recorded from normal joints, the $B_2$ receptor antagonist, Hoe 140 (100μgkg$^{-1}$, i.a.), caused insurmountable antagonism of the BK(1-100μg)-induced increase in spontaneous discharge, but caused surmountable antagonism of the BK(1-100μg)-induced sensitisation of mechanonociceptors to mechanical stimuli (Figure 7.16).

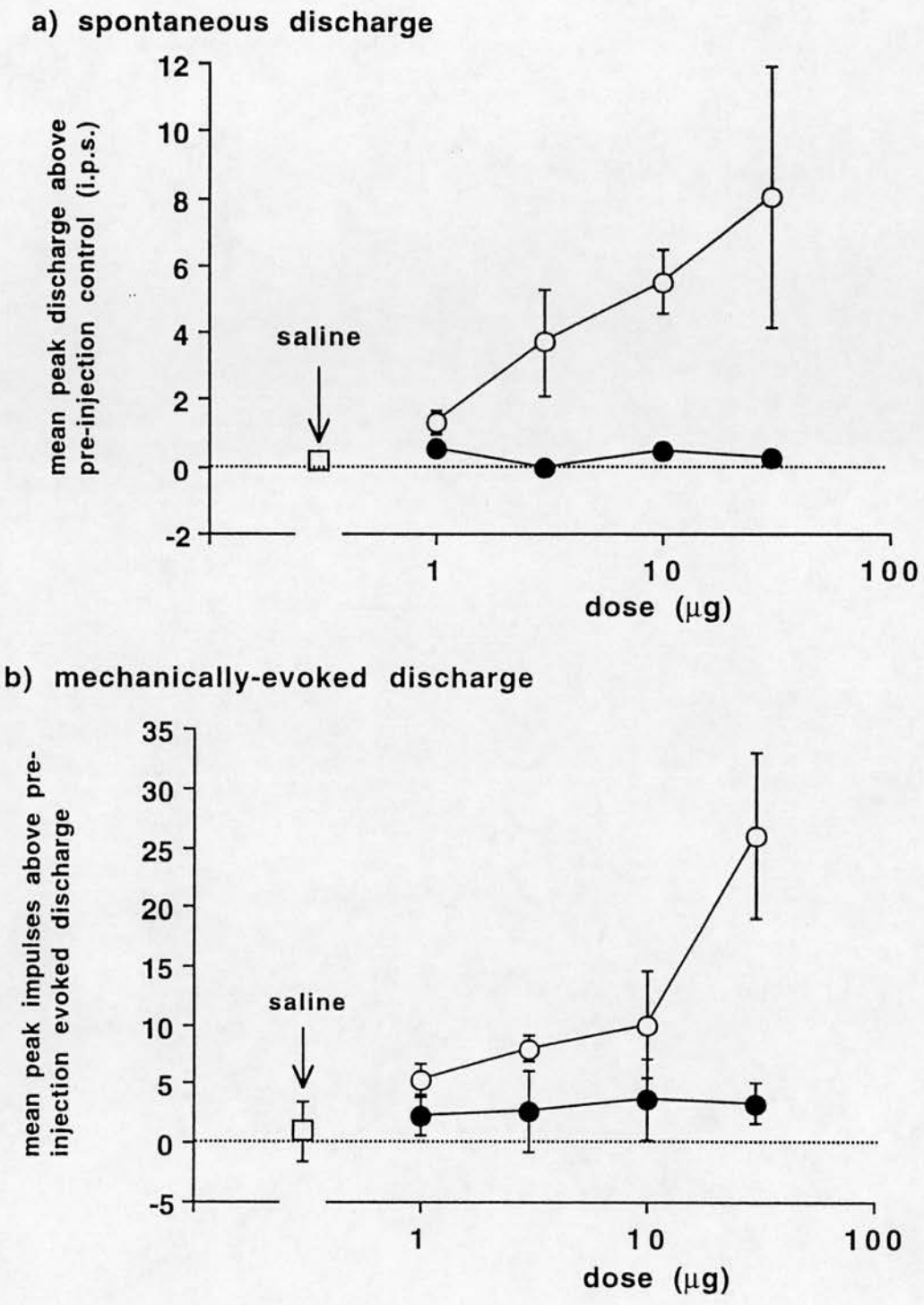



Figure 7.12 Comparison of bradykinin ( ○ ) and des-Arg$^9$-BK ( ● ) on (a) spontaneous and (b) mechanically-evoked discharge from acutely-arthritic joints (3-5 days post-adjuvant). Each point is the mean ± s.e.mean from 2 - 3 units (2 - 3 individual experiments).

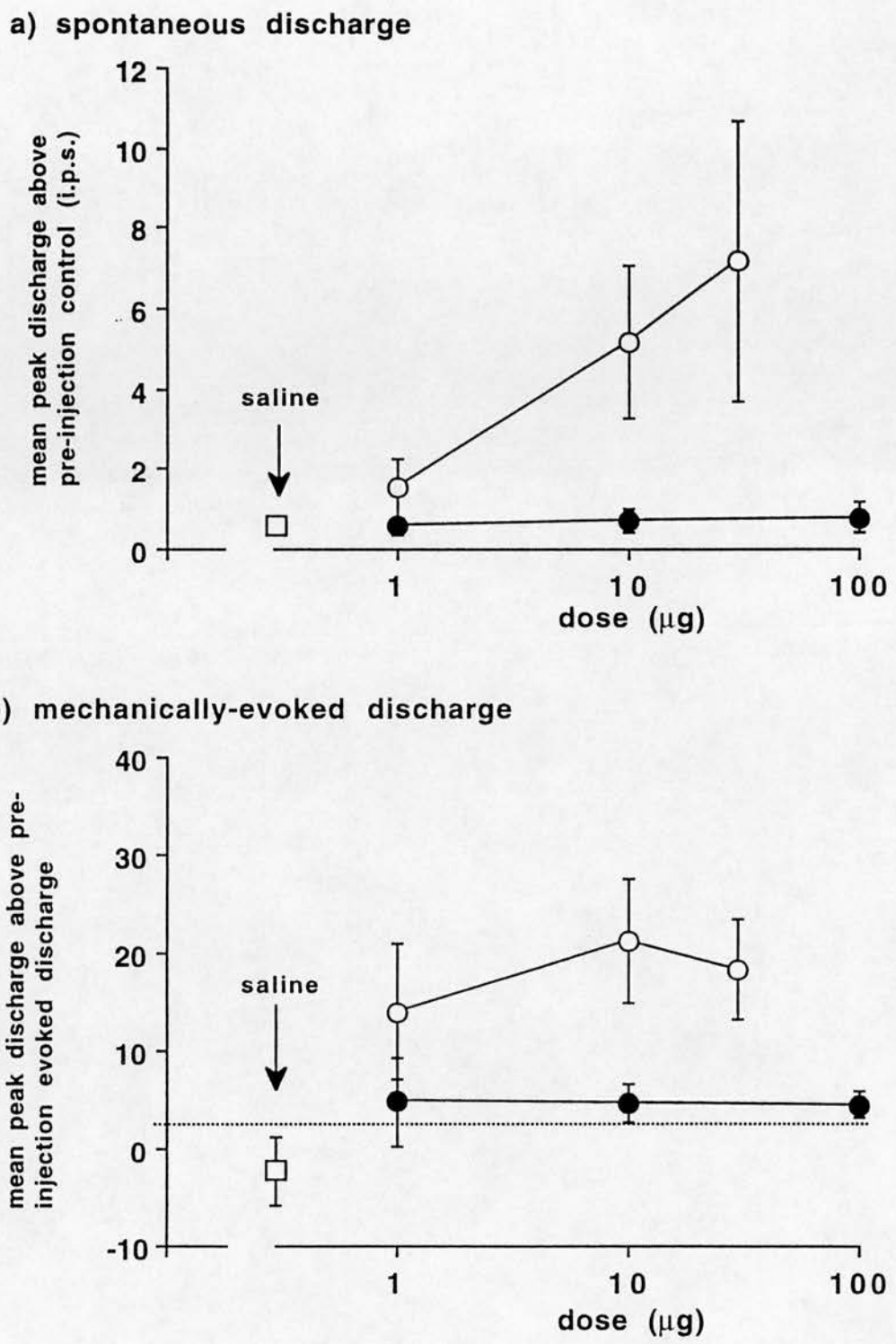



Figure 7.13 Comparison of bradykinin ( ○ ) and des-Arg$^9$-BK ( ● ) on (a) spontaneous and (b) mechanically-evoked discharge from chronically-arthritic joints (19 - 30 days post-adjuvant). Each point is the mean ± s.e.mean from 4 - 6 units (3 - 6 individual experiments).

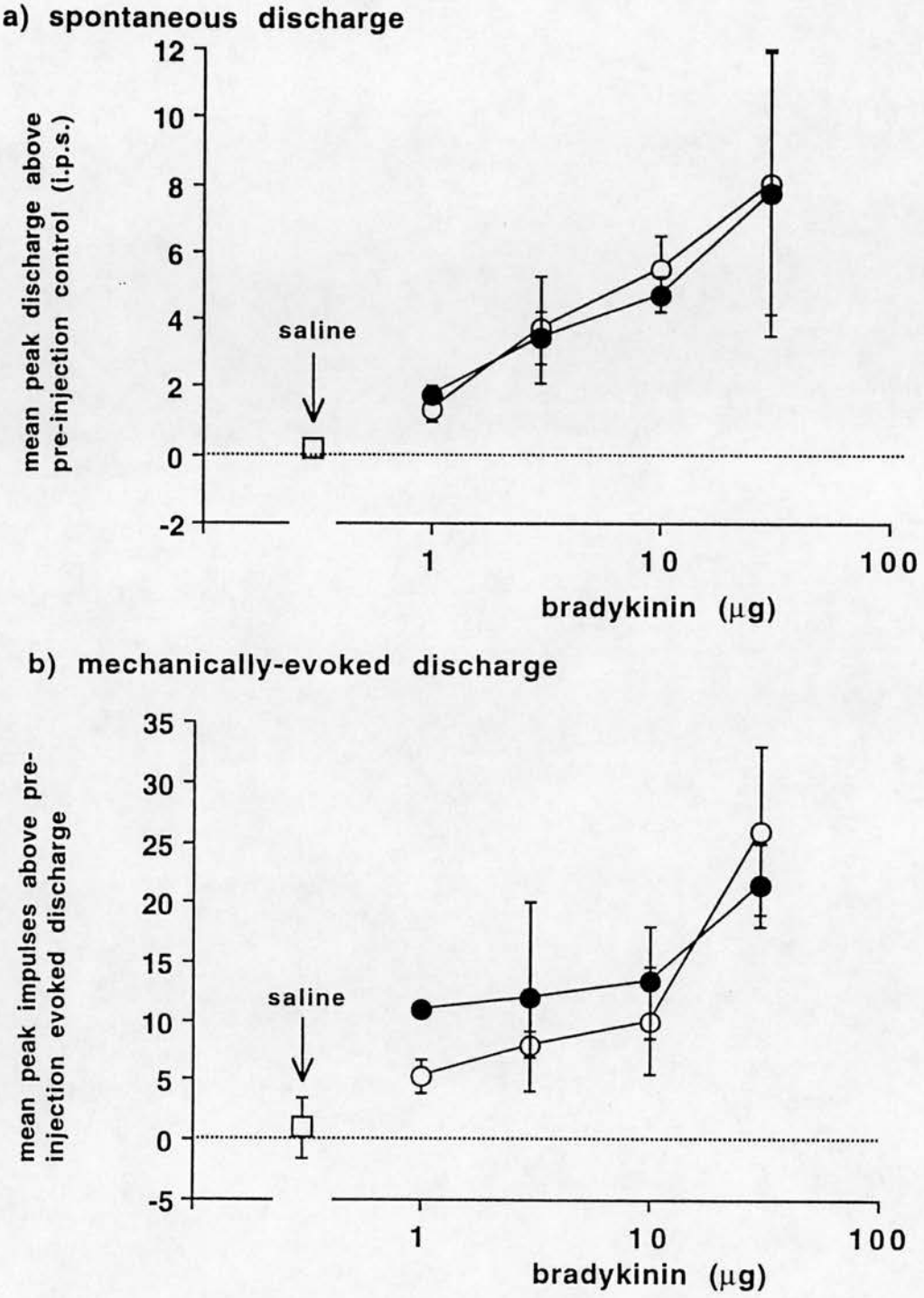



Figure 7.14 Bradykinin-induced enhancement of (a) spontaneous and (b) mechanically-evoked discharge before ( ○ ) and after ( ● ) des-Arg$^{9}$-Leu$^{8}$-BK (1mgkg$^{-1}$, i.a.) in acutely-arthritic joints (3 - 5days post-adjuvant). Each point is the mean ± s.e.mean from 2 - 3 units (2 - 3 individual experiments).

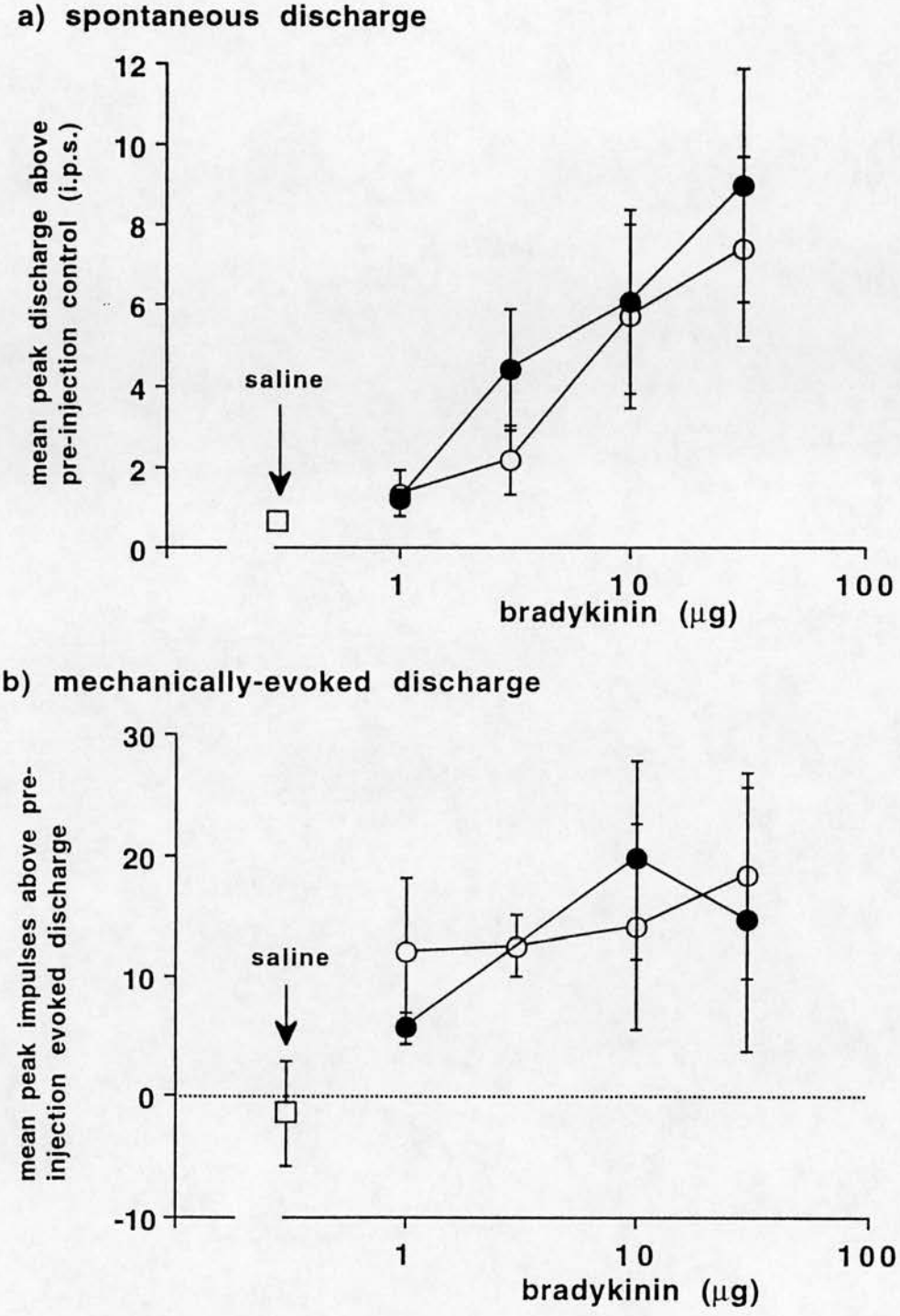



Figure 7.15 Bradykinin-induced enhancement of (a) spontaneous and (b) mechanically-evoked discharge before ( ○ ) and after ( ● ) des-Arg$^{9}$-Leu$^{8}$-BK (1mgkg$^{-1}$, i.a.) in chronically-arthritic joints (15 - 26 days post-adjuvant). Each point is the mean ± s.e.mean from 4 - 5 units (3 - 5 individual experiments).

**Table 7.1 Lack of effect of bradykinin receptor antagonists on spontaneous discharge from articular mechanonociceptors in normal rat ankle joints.**

| | *units examined (n)* | *impulses above basal discharge* | *† P* | *impulses below basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 9 (9) | 6 ± 2 | - | 6 ± 3 | - |
| des-Arg$^9$-Leu$^8$-BK 100μgkg$^{-1}$, i.a. | 4 (4) | 5 ± 2 | NS | 4 ± 2 | NS |
| Hoe140 100μgkg$^{-1}$, i.a. | 6 (6) | 5 ± 1 | NS | 6 ± 2 | NS |

† Statistical comparisons (Mann-Whitney U-test) between saline and the test bradykinin receptor antagonist. NS = P>0.05. The pre-injection discharge, was 1.2 ± 0.3 i.p.s pre-saline, 1.4 ± 0.5 i.p.s. pre-des-Arg$^9$-Leu$^8$-BK and 1.6 ± 0.5 i.p.s. pre-Hoe140.

**Table 7.2 Lack of effect of bradykinin receptor antagonists on mechanically-evoked discharge from articular mechanonociceptors in normal rat ankle joints.**

| | *units examined (n)* | *impulses above evoked basal discharge* | *† P* | *impulses below evoked basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 8 (8) | 1.4 ± 0.2 | - | 1.1 ± 0.3 | - |
| des-Arg$^9$-Leu$^8$-BK 100μgkg$^{-1}$, i.a. | 3 (3) | 1.0 ± 0.2 | NS | 1.1 ± 0.2 | NS |
| Hoe140 100μgkg$^{-1}$, i.a. | 5 (5) | 1.1 ± 0.1 | NS | 1.1 ± 0.3 | NS |

† Statistical comparisons (Mann-Whitney U-test) between saline and the test bradykinin receptor antagonist. NS = P>0.05. The pre-injection mechanically-evoked discharge, was 54 ± 9 impulses pre-saline, 48 ± 11 impulses pre-des-Arg$^9$-Leu$^8$-BK and 52 ± 12 impulses pre-Hoe140.

**Table 7.3 Lack of effect of bradykinin receptor antagonists on spontaneous discharge from articular mechanonociceptors in arthritic rat ankle joints.**

| | *units examined (n)* | *impulses above basal discharge* | *† P* | *impulses below basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 9 (9) | 5 ± 2 | - | 6 ± 3 | - |
| des-Arg$^9$-Leu$^8$-BK 100μgkg$^{-1}$, i.a. | 4 (4) | 4 ± 2 | NS | 3 ± 1 | NS |
| Hoe140 10μgkg$^{-1}$, i.a. | 6 (6) | 5 ± 3 | NS | 5 ± 3 | NS |
| Hoe140 100μgkg$^{-1}$, i.a. | 6 (6) | 6 ± 2 | NS | 4 ± 1 | NS |

*†* Statistical comparisons (Mann-Whitney U-test) between saline and the test bradykinin receptor antagonist. NS = P>0.05. The pre-injection discharge, was 2.6 ± 0.7 i.p.s pre-saline, 2.8 ± 0.9 i.p.s. pre-des-Arg$^9$-Leu$^8$-BK, 3.3 ± 1.2 i.p.s. pre-Hoe140 (10μgkg$^{-1}$) and 3.1 ± 0.7 i.p.s. pre-Hoe140 (100μgkg$^{-1}$).

**Table 7.4 Lack of effect of bradykinin receptor antagonists on mechanically-evoked discharge from articular mechanonociceptors in arthritic rat ankle joints.**

| | *units examined (n)* | *impulses above evoked basal discharge* | *† P* | *impulses below evoked basal discharge* | *† P* |
|---|---|---|---|---|---|
| saline | 8 (8) | 1.3 ± 0.3 | - | 1.1 ± 0.2 | - |
| des-Arg$^9$-Leu$^8$-BK 100μgkg$^{-1}$, i.a. | 3 (3) | 0.9 ± 0.2 | NS | 1.4 ± 0.2 | NS |
| Hoe140 10μgkg$^{-1}$, i.a. | 5 (5) | 1.2 ± 0.2 | NS | 1.4 ± 0.2 | NS |
| Hoe140 100μgkg$^{-1}$, i.a. | 5 (5) | 1.3 ± 0.3 | NS | 1.1± 0.2 | NS |

*†* Statistical comparisons (Mann-Whitney U-test) between saline and the test bradykinin receptor antagonist. NS = P>0.05. The pre-injection mechanically-evoked discharge, was 41 ± 7 impulses pre-saline, 43 ± 10 impulses pre-des-Arg$^9$-Leu$^8$-BK, 32 ± 8 impulses pre-Hoe140 (10μgkg$^{-1}$) and 46 ± 6 impulses pre-Hoe140 (100μgkg$^{-1}$).

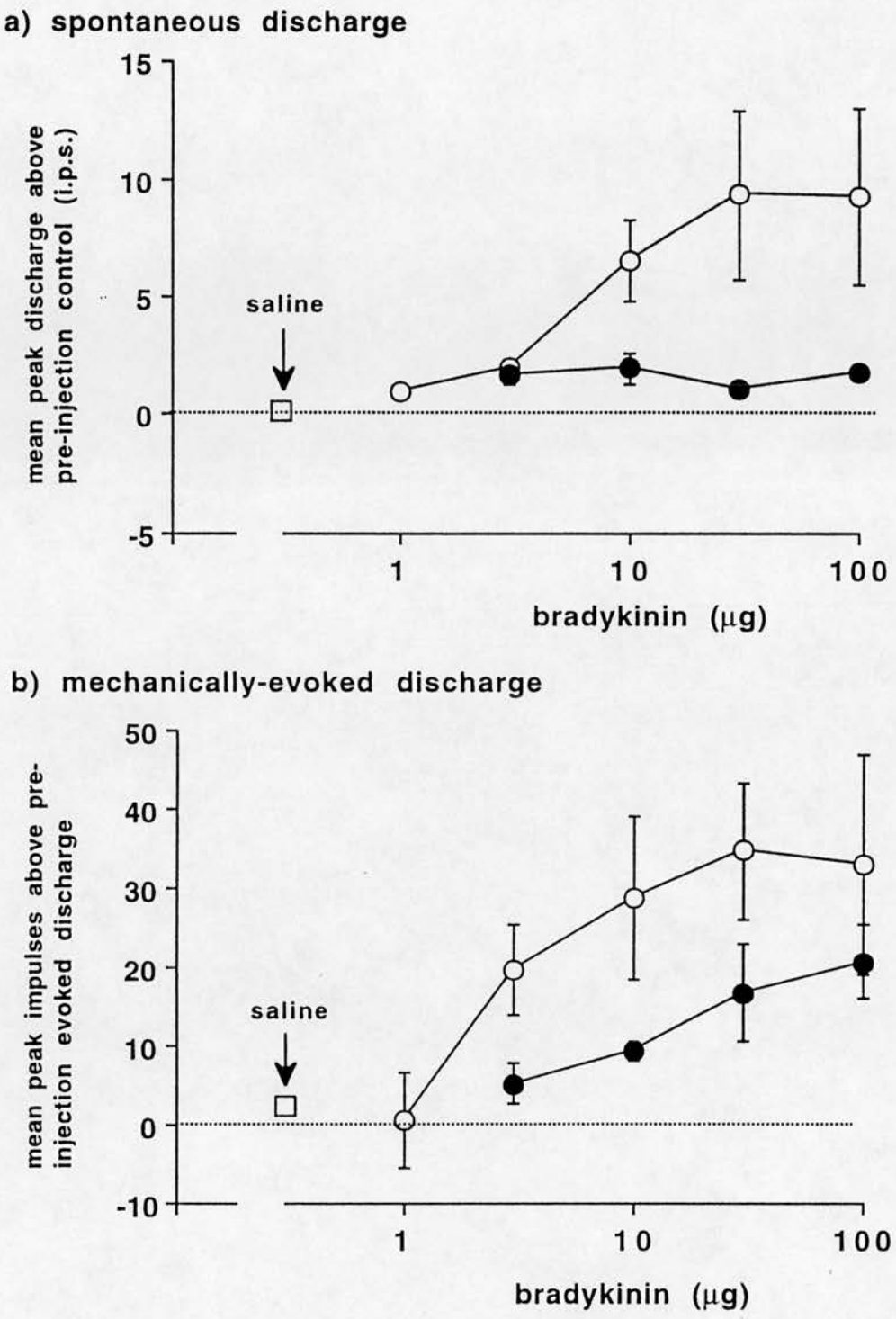



Figure 7.16 Effects of bradykinin on (a) spontaneous and (b) mechanically-evoked discharge before ( ○ ) and after ( ● ) Hoe140 (100μgkg$^{-1}$, i.a.) in normal joints. Each point is the mean ± s.e.mean from 5 - 6 units (4 - 6 individual experiments).

#### 7.3.8.2 Effects of Hoe140 on bradykinin-induced responses in arthritic joints

In units recorded from arthritic joints, BK(1 - 30μg)-induced enhancements in spontaneous discharge were antagonised by Hoe140 (10 and 100μgkg$^{-1}$, i.a.) in an insurmountable manner, whereas BK(1-30μg)-induced sensitisations of mechanonociceptors to mechanical stimuli were antagonised by Hoe140 in a dose-dependent surmountable manner (Figure 7.17).

#### 7.3.8.3 Lack of effect of Hoe140 on spontaneous and mechanically-evoked discharge in normal and arthritic joints

In recordings from normal joints, Hoe140 (100μgkg$^{-1}$, i.a.) had no significant effect on spontaneous (Table 7.1) or mechanically-evoked (Table 7.2) discharge. In units recorded from arthritic joints, Hoe140 (10 & 100μgkg$^{-1}$, i.a.) also had no significant effect on spontaneous discharge (Table 7.3) or on the responsiveness to the standard mechanical stimulus (Table 7.4).

### 7.3.9 Blood pressure studies

As well as having effects on afferent neural discharge, BK (1-100μg, i.a.) also caused a dose-dependent decrease in mean arterial blood pressure in normal and arthritic rats (Figure 7.18 illustrates typical experiments). These BK-induced falls in mean blood pressure were antagonised by the $B_2$ receptor antagonist, Hoe140, but not the $B_1$ receptor antagonist, des-Arg$^9$-Leu$^8$-BK (Figure 7.18).

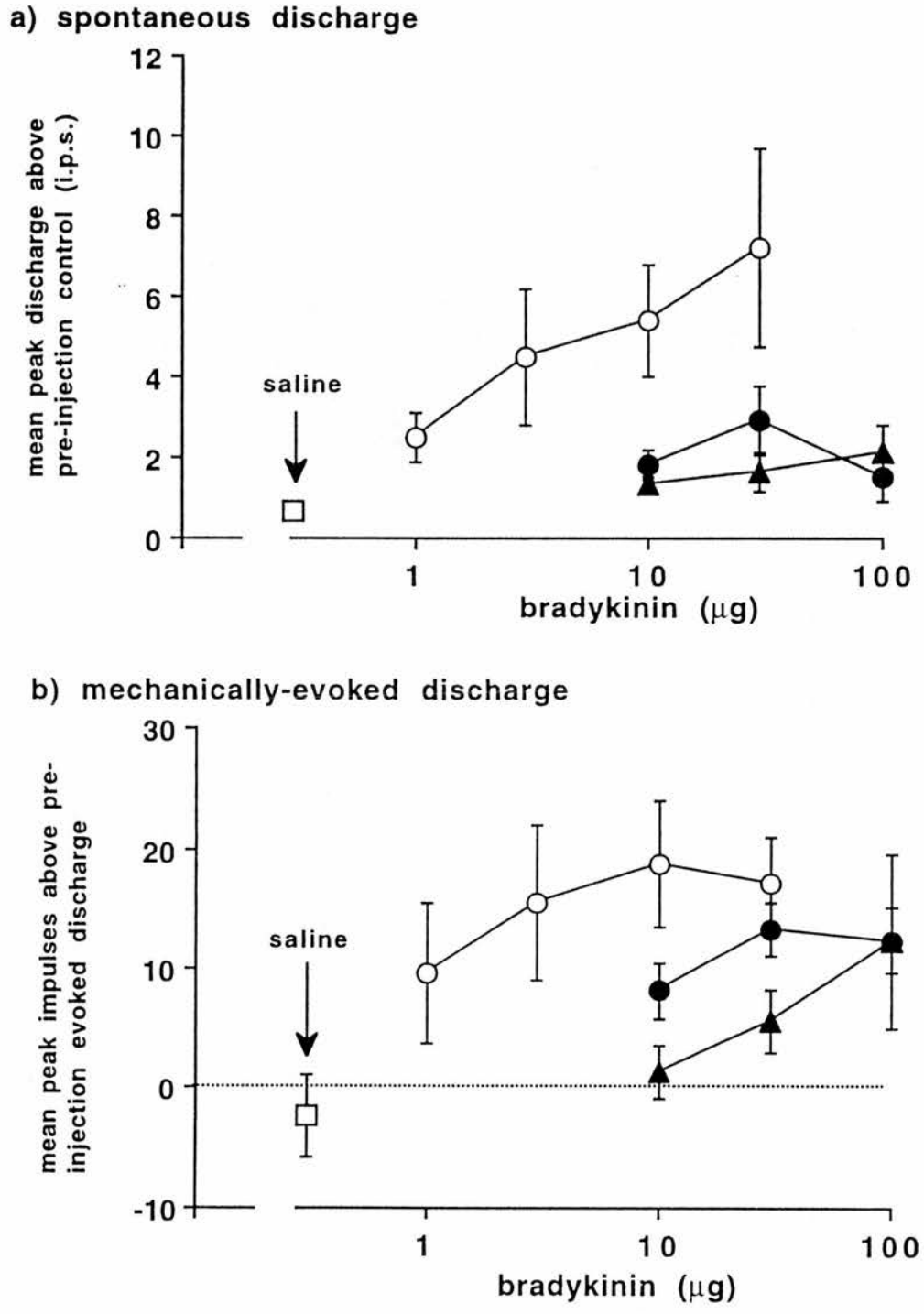



Figure 7.17 Effects of bradykinin on (a) spontaneous and (b) mechanically-evoked discharge before ( ○ ) and after Hoe140 ( ● 10 and ▲ 100μgkg$^{-1}$, i.a.) in chronically-arthritic joints (19 -31 days post-adjuvant). Each point is the mean ± s.e.mean from 5 - 10 units (5 - 8 individual experiments).

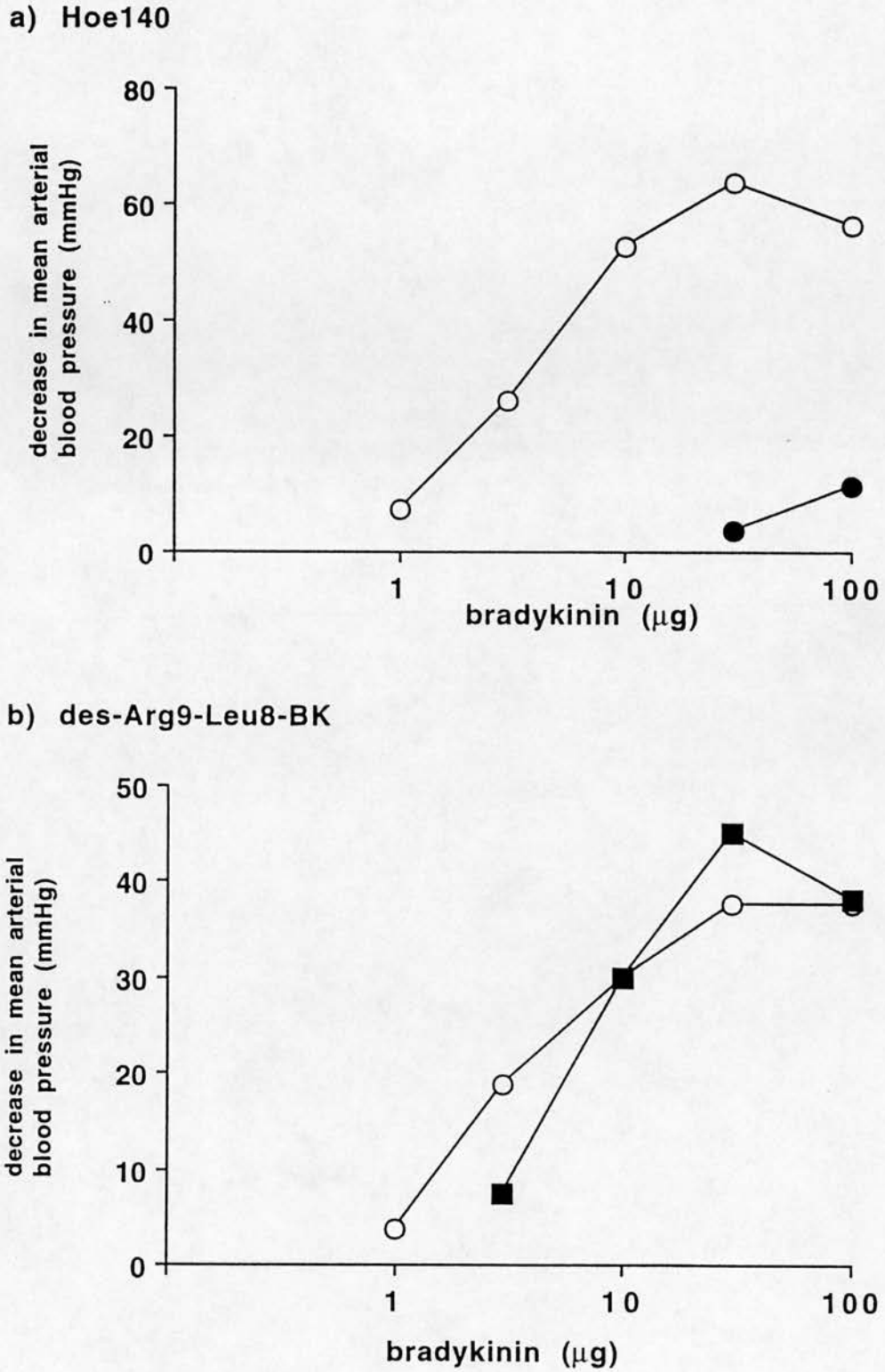



Figure 7.18 Typical single experiments illustrating (a) Hoe140 ( ● )-induced antagonism and (b) lack of effect of des-Arg$^{9}$-Leu$^{8}$-BK ( ■ ) on bradykinin-evoked hypotension ( ○ ) in the normal rat. The data shown in (a) and (b) was from different rats. Similar results were also obtained in arthritic rats (data not shown).

In some of the neural recordings (normal and arthritic joints) which were responsive to BK, and in which Hoe140 was not administered, the non-selective adenosine receptor agonist, 5'-$N^6$-ethylcarboxamidoadenosine (NECA), was injected (10-30μg, i.a.) at the end of the experiment. NECA, like BK, caused marked decreases in mean blood pressure (approximately 40-50mmHg), but unlike BK, produced no change in neural discharge (see Section 5).

## 7.4 DISCUSSION

### 7.4.1 Effects of bradykinin on neural discharge from normal ankle joint mechanonociceptors

#### 7.4.1.1 Bradykinin-induced excitation

In the present study, BK enhanced spontaneous afferent discharge (excitation response) from C-fibre fine afferents innervating normal rat ankle joints in 86% of the units studied. This percentage is similar to the findings by Kanaka et al (1985) in the cat knee joint, where BK excited 92.5% of C- and Aδ-fibre afferent units, and with investigations in various non-articular fine afferents - 86.5% in dog muscle (Kumazawa & Mizumura, 1977); 80% in cat heart (Baker et al., 1980); 93% in dog testis (Kumazawa & Mizumura, 1980); 73% of cat visceral (gallbladder and liver) fibres (Longhurst et al., 1984); 100% in guinea-pig trachea (Fox et al., 1993); 80% in cat cornea (Belmonte et al., 1994).

The doses of intra-arterially injected BK required to produce excitation in the present studies were in the 1-10μg range. A similar dose-range (intra-arterial injection) was also required to cause BK-induced excitation of articular (Kanaka et al., 1985) and visceral (Haupt et al., 1983) afferents in the cat.

The results of the current study show that the delay to the onset of BK-induced excitation was 3 - 150s. Similar delay times to the onset of BK-induced excitation have also been reported in afferents innervating cat knee joints (16s; Kanaka et al.,

1985), dog muscle (28s; Kumazawa & Mizumura, 1977) and rat skin (30s; Lang et al., 1990). In the present study, the mean delay of BK-induced excitation was decreased to approximately 10s at higher doses of bradykinin (10 and 30μg)(Figure 7.3). This observation is consistent with the study by Mense & Schmidt (1974) in cat muscle afferents, where the higher dose of BK (26μg) had a delay to the onset of excitation of only 8.8s.

The results of the present investigation in normal joints have shown that the mean duration of BK-induced excitation varied according to the dose of BK used. At the two lower doses of BK (1 and 3μg) the mean duration was approximately 100s, but at higher doses (10 and 30μg) was increased by approximately 2 fold (Figure 7.4). Thus, increasing the dose of BK results in a longer action of the kinin. This is in contrast with the results obtained in rat skin afferents where increases in BK concentration did not change the duration of the response (Lang et al., 1990). Although somewhat variable, duration of BK responses of 12-138s, 14-167s, and 30-300s were observed from afferents in cat muscle (Mense & Schmidt, 1974), rabbit ear (Szolcsányi, 1987) and rat skin (Lang et al., 1990), respectively. These durations are comparable with the findings of the present study.

#### 7.4.1.2 Bradykinin-induced mechanonociceptor sensitisation

In the current study, BK increased the responsiveness of mechanonociceptors to mechanically-evoked stimuli. Similar BK-induced sensitisation of mechanoreceptors to mechanically-evoked stimuli have also been reported in canine hearts (Uchida &

Murao, 1974), cat muscle (Mense & Mayer, 1987), and in cat knee (Neugebauer et al., 1989) or rat ankle (Grubb et al., 1991; Birrell et al., 1993) joints. Using thermal stimuli, BK-induced sensitisation of mechano-heat-sensitive units has also been demonstrated in the skin of cats (Beck & Handwerker, 1974) and rats (Koltzenberg et al., 1992).

In the present experiments, BK-induced enhancement in the responsiveness to mechanical stimuli was observed in 80% of the C-fibre afferent units recorded. This percentage compares fairly well with the proportions found in cat muscle afferents (38% C-fibres and 67% Aδ-fibres; Mense & Mayer, 1987), and in afferents from cat knee joints (70%; Neugebauer et al., 1989).

### 7.4.2 Bradykinin-induced excitation and sensitisation in arthritic joints

The current investigation has shown that BK induced an increase in spontaneous discharge (excitation), and enhanced the responsiveness to mechanical stimuli (sensitisation), of articular mechanonociceptors in chronically inflamed (adjuvant-arthritic) ankle joints. BK induced excitation in 89% of units, and sensitisation of 88%. These percentages were similar to those obtained in units from normal joints (see 7.4.1.1 & 7.4.1.2).

Various investigators have reported that sensitivities of nociceptors are enhanced under inflammatory conditions (Coggeshall et al., 1983; Guilbaud et al., 1985; Schaible & Schmidt, 1985; Grigg et al., 1986; Schaible & Schmidt, 1988). One group

of mediators that may be responsible for this enhanced sensitivity of nociceptors are the prostanoids. Indeed, it has been shown that levels of prostanoids are raised in arthritic joints (Robinson et al., 1975; Trang et al., 1977), and that they can potentiate BK-induced excitation and sensitisation (Grubb et al., 1991; Schepelmann et al., 1992; Birrell et al., 1993). From these observations, it would have been expected that in the present investigation BK-induced excitation and sensitisation in normal joints would be enhanced in joints with chronic inflammation (adjuvant-arthritis). However, BK-induced excitation (10 and 30μg) and sensitisation (3, 10 and 30μg) in arthritic joints were smaller in magnitude (although this difference did not reach statistical significance) than that obtained from the ankle joints of normal rats. The are several hypotheses which could explain the smaller responses to BK in arthritic joints. One is that since BK levels are elevated in inflammatory states (Lewis, 1970; Hargreaves & Costello, 1990), then BK will have to act on an elevated baseline such that the increase induced by BK will be apparently reduced. However, this hypothesis is not supported by the studies which showed that bradykinin receptor ($B_1$ and $B_2$) antagonists had no effect on basal spontaneous or mechanically-evoked discharge in arthritic joints (see Section 7.3.7.3 and 7.3.8.3). A desensitisation or down regulation of BK receptors with the development of chronic arthritis may explain the reduced responsiveness of BK in arthritic joints. Another possible explanation to account for the reduced BK responses is the observation that BK can cause the release of opioids (Kudo et al., 1986). Since opioids have been shown to reduce afferent discharge from inflamed knee joints (Russell et al., 1987), then it is possible that the reduced responses to BK observed in the present study are due to the evoked release of opioids by BK. Therefore, in arthritic joints the responses to BK may be a

combination of an excitatory action of BK and an inhibitory action of released opioids. This hypothesis could be tested by determining the effects of bradykinin before and after opioid receptor antagonists such as naloxone. Regarding the possible location of action of peripheral opioids, electrophysiological recordings from fine afferent fibres from the inflamed (kaolin and carrageenan-induced) cat knee joint have indicated that opiates act on opioid receptors (mu and kappa) located at peripheral sites of primary afferent fibres to cause a peripheral analgesic effect (Russell et al., 1987). The reduction in mechanical hyperalgesia in adjuvant-arthritic rats by the local (but not the same dose given systemically) injection of the opioid, fentanyl, also suggests a role for peripherally located opioid receptors (mu subtype) in the modulation of nociception in inflamed tissue (Stein et al., 1988). The possible sources of endogenous opioid ligands for the peripheral receptors are the primary afferent neurone (Weihe, 1989), pituitary-adrenal axis (Vale et al., 1981; Milan and Herz, 1985) or the immune system (Sibinga & Goldstein, 1988).

Recently, in our laboratory we have shown that BK-induced excitation in arthritic joints was significantly greater following $N^G$--nitro-arginine-methyl-ester (L-NAME) injection (Kelly et al., 1994). This finding suggests that nitric oxide may be responsible for the reduced responses to BK in the arthritic joint. In support of this is the study by Dray et al., (1992) in the neonatal spinal cord-tail preparation where bradykinin-induced depolarisation was reduced in nitroprusside-treated preparations.

In arthritic joints, the mean duration (approximately 100s) and delay (approximately 40s) of BK-evoked excitatory responses were not altered with increasing dose of BK.

In contrast, increasing the dose of BK caused reductions in the onset of excitation, as well as increasing response duration in units recorded from normal joints. It is unclear as to why these differences in duration and delay times arose between normal and arthritic joints, but may have been due to the action of released opioids by BK in arthritic joints.

### 7.4.3 Independence of bradykinin-induced excitation and sensitisation

In agreement with the articular afferent neural recordings by Neugebauer et al (1989) in the cat, most units in the current study were both excited and sensitised by BK. However, in the present study (normal and arthritic joints) it was possible to have a BK-induced excitation without altering the responsiveness to mechanically-evoked stimuli. Furthermore, although BK induced a dose-dependent increase in mechanically-evoked discharge, there was desensitisation of BK-evoked excitation at higher doses. Moreover, the duration of BK-induced sensitisation could be greater than that of BK-induced excitation or vice-versa (Figure 7.7). From these observations it can be concluded that there is no correlation between BK-induced excitation and sensitisation, and that these responses are probably independent of each other. It can be further hypothesised that bradykinin-induced excitation and sensitisation of ankle joint mechanonociceptors may occur via different mechanisms. For example, BK-evoked excitation and sensitisation may involve different second messenger systems. Indeed, in sensory tissues, protein kinase C is involved in BK-induced excitation (Dray et al., 1988; Burgess et al., 1989), whereas BK can cause inhibition of a slow after-hyperpolarisation (Weinreich, 1986; Weinreich & Wonderlin,

1987), an effect mediated by cyclic-AMP, so as to increase neural excitability. Another possibility to account for the independent occurrence of BK-induced excitation and sensitisation is that, whereas BK-induced excitation is due to a direct action of BK on afferent fibres, BK-induced sensitisation could be mediated indirectly by the release of mediators from leukocytes, mast cells, macrophages or platelets such as prostanoids, leukotrienes or 5-HT.

### 7.4.4 Extent of desensitisation of bradykinin-induced excitation and sensitisation

Desensitisation of BK-induced excitation and sensitisation, in terms of repeat dose (10μg) or increasing dose, was generally not observed in units recorded from either normal or arthritic joints in the present investigation. This is in contrast to the marked desensitisation of BK-induced enhancements in neural discharge from fine afferent fibres in the cat knee joint (Kanaka et al., 1985), dog testis (Kumazawa et al., 1987) or rat skin (Lang et al., 1990). In line with the present results, desensitisation to BK was not reported in afferents innervating dog viscera (Guzman et al., 1962), cat skeletal muscle (Hiss & Mense, 1976; Mense et al., 1982) and cat cornea (Belmonte et al., 1994).

### 7.4.5 Effects of indomethacin on bradykinin-induced excitation and sensitisation

The results of the present study have shown that BK-evoked excitation or sensitisation of mechanonociceptors in either normal or arthritic joints was not affected by indomethacin. This then strongly suggests that BK does not release

prostaglandins to mediate BK-induced responses in ankle joint mechanonociceptors. In contrast, BK-induced responses in muscle, visceral and cutaneous nociceptors have been shown to be dependent on prostanoids, as the responses to BK were reduced by aspirin, paracetamol or indomethacin (Kumazawa & Mizumura, 1980; Mense, 1982; Dray et al., 1992). Nevertheless, in agreement with the present study, it has been demonstrated that BK-induced excitation of vagal afferents was not dependent on prostaglandins (Fox et al., 1993). Although the results of the present investigation suggest that BK-induced responses do not involve prostanoids, it is possible that they may still be secondary to release by BK of other mediators such as leukotrienes (Samuelsson, 1983), calcitonin gene-related peptide (Franco-Cereceda et al., 1989), nitric oxide (Ignarro et al., 1986) or cytokines (Ferreira et al., 1993).

### 7.4.6 Afferent neural discharge and blood pressure

Although BK and the non-selective adenosine receptor agonist, NECA, both decreased mean arterial blood pressure, it was only BK that affected discharge of articular afferents. This implies that BK-induced alterations in mechanonociceptor discharge were not a result of the hypotensive action of BK.

### 7.4.7 $B_1$ receptor studies on ankle joint mechanonociceptors

#### Normal joints

The results of the present investigation have shown that the $B_1$ receptor agonist, des-Arg$^9$-BK, did not alter basal spontaneous or mechanically-evoked discharge, and that the $B_1$ receptor antagonist, des-Arg$^9$-Leu$^8$-BK, had no effect on BK-induced

excitation or sensitisation. These results, therefore, show that $B_1$ receptors are unlikely to be involved in affecting discharge from articular mechanonociceptors in normal joints. Other studies have also shown non-responsiveness to $B_1$ receptor agonists and antagonists in non-inflamed tissues (Farmer et al., 1991a; Perkins & Kelly, 1993; Perkins et al., 1993; Davis & Perkins, 1994). However, in contrast to these studies and to the present investigation, blockade of BK actions was achieved by des-Arg$^9$-Leu$^8$-BK in 37% of units recorded from articular afferents in normal cat knee joints, indicating the involvement of $B_1$ receptors in the BK response (Messlinger et al., 1992).

**Arthritic joints**

In contrast to the lack of involvement of $B_1$ receptors in normal tissues, it has been shown that under inflammatory situations $B_1$ receptors become involved. For example, in models of persistent inflammatory Freunds adjuvant-induced mechanical (Perkins et al., 1993; Davis & Perkins, 1994) or ultra violet light-induced thermal (Perkins & Kelly, 1993; Perkins et al., 1993) hyperalgesia, des-Arg$^9$-BK produces significant hyperalgesia which is antagonised by des-Arg$^9$-Leu$^8$-BK. In view of such evidence supporting the involvement of $B_1$ receptors under persistent inflammatory situations, it might be expected that, since the present investigation also used a persistent model of inflammation (Freunds adjuvant-induced arthritis), $B_1$ receptor agents would influence neural discharge (excitation and / or sensitisation) from mechanonociceptors in arthritic joints. However, the results obtained in chronically-arthritic rats (15-30 days post-adjuvant) showed that des-Arg$^9$-BK had no effect on articular afferent neural discharge, and that BK-induced excitation and sensitisation

were not affected by des-Arg$^9$-Leu$^8$-BK. Further evidence which indicates that $B_1$ receptors are not involved in modulating articular afferent discharge in inflamed joints, is the finding that des-Arg$^9$-Leu$^8$-BK did not affect basal spontaneous or mechanically-evoked discharge in arthritic joints (Tables 7.3 - 7.4). In agreement it has been shown that des-Arg$^9$-Leu$^8$-BK also does not affect the ongoing afferent activity from inflamed (kaolin and carrageenan-induced) cat knee joints (Meblinger et al., 1993).

It is unclear as to why $B_1$ receptors play a role in the persistent hyperalgesia in the studies by Perkins & Kelly (1993) and Davis & Perkins (1994) but appear not to be involved in modulating neural discharge in rats also with a persistent inflammatory hyperalgesia (adjuvant-arthritis) in the current investigation. Differences between the times at which $B_1$ receptor agents were examined in the study by Davis & Perkins (1994) (3-4 days post-adjuvant) and the present investigation (15 - 30 days post-adjuvant) cannot explain the differences observed since the present study also demonstrated lack of activity for $B_1$ receptor agonists and antagonists 3 - 5 days post-adjuvant. Other possibilities which could explain the difference in the responsiveness to $B_1$ receptor agonists / antagonists between the present study and those of Perkins & Kelly (1993) and Davis & Perkins (1994) are the different models (behavioural versus electrophysiological) used, or the age, sex, and species of rats used.; young (80-100g) female Sprague-Dawley rats were used by Perkins & Kelly (1993) and Davis & Perkins (1994), whereas the studies in the present investigation used adult (350-450g) male Wistar rats.

### 7.4.8 Effects of Hoe140

#### 7.4.8.1 Actions of Hoe140 on BK-induced responses in normal and arthritic joints

The potent (Bao et al., 1991; Lembeck et al., 1991; Wirth et al., 1991) bradykinin $B_2$ receptor antagonist, Hoe140, antagonised BK-induced excitation and sensitisation in both normal and arthritic joints. In agreement with these results of the present investigation, antagonism of BK-induced responses by Hoe140, consistent with the involvement of $B_2$ receptors, has also been reported in various models of hyperalgesia and inflammation (Lembeck et al., 1991; Wirth et al., 1991; Damas & Remacle-Volon, 1992; Messlinger et al., 1992; Heapy et al., 1993; Davis & Perkins, 1994).

In the present study, Hoe140 showed a differing profile of antagonism for BK-evoked responses; surmountable antagonism of BK-induced sensitisation, and apparently insurmountable antagonism of BK-induced excitation. This surmountable / insurmountable nature of Hoe140 is also observed in the rat vas deferens (see Section 9), and in cultured colonic epithelial cells (Cuthbert et al., 1992). One conclusion that can be drawn from this differential response of Hoe140 is that the $B_2$ receptors responsible for BK-induced excitation and sensitisation are not of a homologous nature. There may be present subtypes of the $B_2$ receptor or different types of BK receptor such as $B_3$, $B_4$ or $B_5$ receptor. However, caution is required when interpreting the actions of Hoe140 since, as discussed in detail for the rat vas deferens in Section 9.3.8, the differential actions of Hoe140 may simply be reflections of tissue

kinetics, or could be explained by other mechanisms not involving subtypes of the BK receptor. In order to provide evidence to support the suggestion for the existence of subtypes of the $B_2$ receptor mediating BK-induced excitation and sensitisation further work using other potent and stable $B_2$ receptor antagonists (e.g. D-Arg-[$Hyp^3$, $Thi^5$, D-$Tic^7$, $Tic^8$]-BK, [$Arg(Tos)^1$, $Hyp^3$, $Thi^5$, D-$Tic^7$, $Oic^8$]-BK, NPC 17731 and NPC 1776; Farmer et al., 1991b; Lembeck et al., 1991; Corrêa & Calixto, 1993) is required in order to determine whether these also behave in a surmountable / insurmountable manner.

### 7.4.8.2 Hoe140 and basal articular mechanonociceptor discharge

As described in Sections 3 and 4 of this thesis, and in electrophysiological studies in rats and cats (Coggeshall et al., 1983; Guilbaud et al., 1985; Schaible & Schmidt, 1985; Grigg et al., 1986; Meblinger et al., 1993; Schaible & Grubb, 1993), articular mechanonociceptors show elevated resting (spontaneous) discharge and sensitisation to mechanical stimuli following inflammation. The present evidence, that normal joint articular mechanonociceptors were excited and / or sensitised by BK, and that these responses could be blocked by the $B_2$ receptor antagonist Hoe140, suggested that endogenous BK acting via $B_2$ receptors could potentially play a role in causing the enhanced ongoing discharge and sensitisation to mechanical stimuli of mechanonociceptors in chronically-arthritic ankle joints. However, the results of the present experiments demonstrated that Hoe140 did not alter articular afferent discharge in arthritic (also normal) rat joints. This suggests that $B_2$ receptors are not likely to be involved in mediating the enhanced neural discharge from

mechanonociceptors in chronically-inflamed (adjuvant- arthritic) joints. In support of this conclusion is the study by Meblinger et al. (1993) who found that Hoe 140 did not affect ongoing discharges from articular afferents innervating inflamed (induced by kaolin and carrageenan) cat knee joints. In contrast with this study, and the present electrophysiological investigations, anti-hyperalgesic or anti-nociceptive activities have been obtained using Hoe140 in various behavioural models of inflammation and hyperalgesia (Wirth et al., 1991; Damas & Remacle-Volon, 1992; Corrêa & Calixto, 1993; Heapy et al., 1993; Sharma et al., 1993; Davis & Perkins 1994). In most of these behavioural studies the effects of Hoe140 was determined >30min post-injection of Hoe140. Therefore, since in the present investigations the effects of Hoe140 were determined over a 10-15min post-injection period, it is possible that this time period was insufficient to observe any changes in neural discharge induced by Hoe140. However, behavioural studies by Heapy et al. (1993) demonstrated that Hoe140 produced antinociceptive effects in less than 5min, indicating that Hoe140 has a fast onset of action. The results in the rat vas deferens also support a fast onset of action of Hoe140 (see Section 9).

In electrophysiogical studies, it was reported by Messlinger et al (1992) that Hoe140 produced a short-lasting excitation of C- and Aδ-fibres innervating the cat knee joint. No such agonistic activity (excitation or sensitisation) of Hoe140 was observed in the present experiments on articular afferents from rat ankle joints (normal or arthritic).

The results of the current neuropharmacological investigation on C-fibres innervating chronically-inflamed (adjuvant-arthritic) rat ankle joints do not support a

therapeutic role for $B_2$ receptor antagonists such as Hoe140 in the treatment of chronic pain and hyperalgesia associated with arthritis. However, a therapeutic role for Hoe140 is suggested from the results of behavioural studies in rats with adjuvant-induced arthritis of the knee joint, where Hoe140 reduces swelling, inflammation, and mechanical hyperalgesia (Sharma, 1993; Davis & Perkins, 1994). Clearly, studies in humans are required in order to determine the importance of Hoe140 as an anti-inflammatory or analgesic drug in the treatment of disorders such as rheumatoid arthritis.

## 7.5 SUMMARY

The results of the present electrophysiological investigation have shown that intra-arterial injection of bradykinin (BK) causes an enhancement in spontaneous (excitation) and mechanically-evoked (sensitisation) discharge from articular mechanonociceptors in normal and arthritic joints. BK-induced responses in normal joints were generally of a greater magnitude, with longer durations and shorter delays, than the responses in arthritic joints. Since indomethacin did not affect BK-induced responses this strongly suggests that prostanoids are not likely to mediate the effects of BK in this model.

Bradykinin $B_1$ receptors appear not to be involved in altering C-fibre discharge in either normal or chronically inflamed joints as 1) the bradykinin $B_1$ receptor agonist, des-Arg$^9$-BK, had no effect, 2) BK-induced responses were unaffected by the $B_1$

receptor antagonist, des-Arg$^9$-Leu$^8$-BK, and 3) des-Arg$^9$-Leu$^8$-BK had no effect on basal spontaneous or mechanically-evoked discharge.

The $B_2$ receptor antagonist, Hoe140, antagonised BK-induced excitation and sensitisation in normal and arthritic joints. Moreover, Hoe140 had a different profile of antagonism; surmountable antagonism of BK-induced sensitisation, whereas insurmountable antagonism of BK-induced excitation. One interpretation of this differential action of Hoe140 is that it is evidence to support the existence of subtypes of the $B_2$ receptor.

Hoe140 had no effect on the elevated spontaneous discharge and the enhanced responsiveness to mechanical stimuli in chronically-arthritic joints. This suggests that endogenous bradykinin, acting via $B_2$ receptors, is unlikely to be involved in the elevated neural discharge associated with adjuvant-arthritic rats.

## *SECTION 8*

## *EFFECT OF BRADYKININ ON RAT NERVES IN-VITRO.*

## 8.1 INTRODUCTION

As shown in the previous section of this thesis, bradykinin (BK) caused excitation, and sensitisation to mechanical stimuli, of mechanonociceptors in both normal and chronically inflamed joints. It is particularly interesting that these responses to BK were blocked differentially by the potent bradykinin $B_2$ receptor antagonist, Hoe 140; surmountable antagonism of BK-evoked sensitisation was obtained, whereas insurmountable antagonism of the BK-induced excitation occurred. Since these neuropharmacological experiments were performed *in-vivo*, it was difficult to interpret the results or to perform further detailed studies of the effects of BK analogues such as Hoe 140. The difficulties associated with *in-vivo* experimentation include the problems of clearly establishing the mechanisms of drug action, for example, because of the possible contribution of blood or tissue borne mediators or the problems of the complex interactions between body systems. To overcome some of these problems, it was decided to investigate the effects of BK and its analogues in a simple *in-vitro* rat nerve ('grease-gap') preparation. In such a preparation, drug-induced depolarisations and/or hyperpolarisations (DC potentials) from whole nerve bundles are recorded extracellularly. The 'grease gap' technique has been used successfully by various investigators to record capsaicin-induced depolarisation of rat vagus (Marsh et al., 1987), 5-HT-induced depolarisation of rat (Ireland, 1987; Ireland & Tyers, 1987;) or rabbit (Elliot et al., 1990; Elliot & Wallis, 1990) vagus, 5-HT-induced hyperpolarisation of rat superior cervical ganglion (Ireland, 1987; Ireland & Jordan, 1987), prostanoid-induced depolarisation of rat or rabbit vagus (Birrell,

1990), and depolarisation of rat superior cervical ganglion induced by the peptide, angiotensin (Hawcock et al., 1992).

The aim of the present study was to determine whether BK or the $B_1$ receptor agonist, des-Arg$^9$-BK, could alter the membrane potential of various rat nerves (vagus, tibial, tibialis, sciatic and saphenous) *in-vitro*, using a 'grease-gap' technique. The rat vagus, tibial, tibialis, sciatic and saphenous nerves were chosen because approxiamtely 50% of the axons are unmyelinated (see Jenq & Coggeshall, 1984; Jenq & Coggeshall, 1985; Schmalbruch, 1986) - the primary articulo-cutaneous ramus nerve, as recorded from in the present neuorpharmacological experiments (see Sections 3 - 7), could not be used as only a very short length (<3mm) of the nerve could be dissected out, which was insufficient for 'grease-gap' recordings.

## 8.2 MATERIALS & METHODS

In brief, adult male Wistar rats (200-350g) were killed by cervical dislocation and segments of vagus, tibial, tibialis, sciatic and saphenous nerve, of approximately 10 - 15mm, were dissected out and mounted across two-compartment perspex baths such that approximately 50% of the nerve lay in the first compartment, while the remainder projected through a greased slot (Dow-Corning high vacuum grease) into the second. Each compartment of the bath was perfused continuously with Krebs solution (27°C) at a rate of 1 - 1.5ml$min^{-1}$ The potential difference between the two compartments was recorded via silver-silver chloride electrodes, connected to the tissue preparation through an agar-saline / filter paper bridge, and displayed on a potentiometric chart recorder (Rikadenki). Drugs (made up in Krebs solution) were applied at known concentration into the perfusate of the first compartment only.

An attempt was made to obtain log concentration-response curves to bradykinin and des-$Arg^9$-bradykinin (0.01- 1000μM). In order to determine whether or not BK receptors are induced in the nerves *in-vitro*, a protocol was devised where a single concentration of bradykinin or des-$Arg^9$-bradykinin (0.1μM) was applied (contact time 3 - 5min) repeatedly over 4 - 5hr. The effects of superfusing a combination of bradykinin (0.1μM) and $PGE_2$ (1μM) was also determined. To confirm that the preparations were bioactive, a depolarising concentration of KCl (10mM - 100mM) was used either before, but more typically, after perfusion of the test drugs. For further details of the experimental setup and protocols see Section 2.4.

## 8.3 RESULTS

### 8.3.1 Effect of KCl on rat isolated nerves

KCl (10mM - 100mM ) evoked depolarisation of all the nerves studied (vagus, 300 - 400μV; tibial, 250 - 350μV; tibialis, 100 - 250μV; sciatic, 150 - 250μV; saphenous, 100 - 200μV) [see Figure 8.1 for typical response].

### 8.3.2 Investigation of the effect of bradykinin and des-Arg$^9$-BK on rat isolated nerves

In none of the isolated nerves (vagus, tibial, tibialis, sciatic and saphenous) examined (n=4 - 9), did either BK or the $B_1$ receptor agonist, des-Arg$^9$-BK, cause a depolarisation or a hyperpolarisation across the concentration range (0.01- 1000μM) tested (Figure 8.1). There was also no change in the membrane potential of any of the isolated nerves when a single concentration of BK or des-Arg$^9$-BK (0.1μM) was superfused approximately every 25min over a period of 4 - 5hr (n=2 - 5).

### 8.3.3 Effect of a combination of bradykinin and $PGE_2$ on rat isolated nerves

$PGE_2$ (1μM), alone or in combination with BK (0.1μM), had no effect on the membrane potential of any of the nerves (vagus, tibial, tibialis; n=2 - 4) investigated (Figure 8.1).

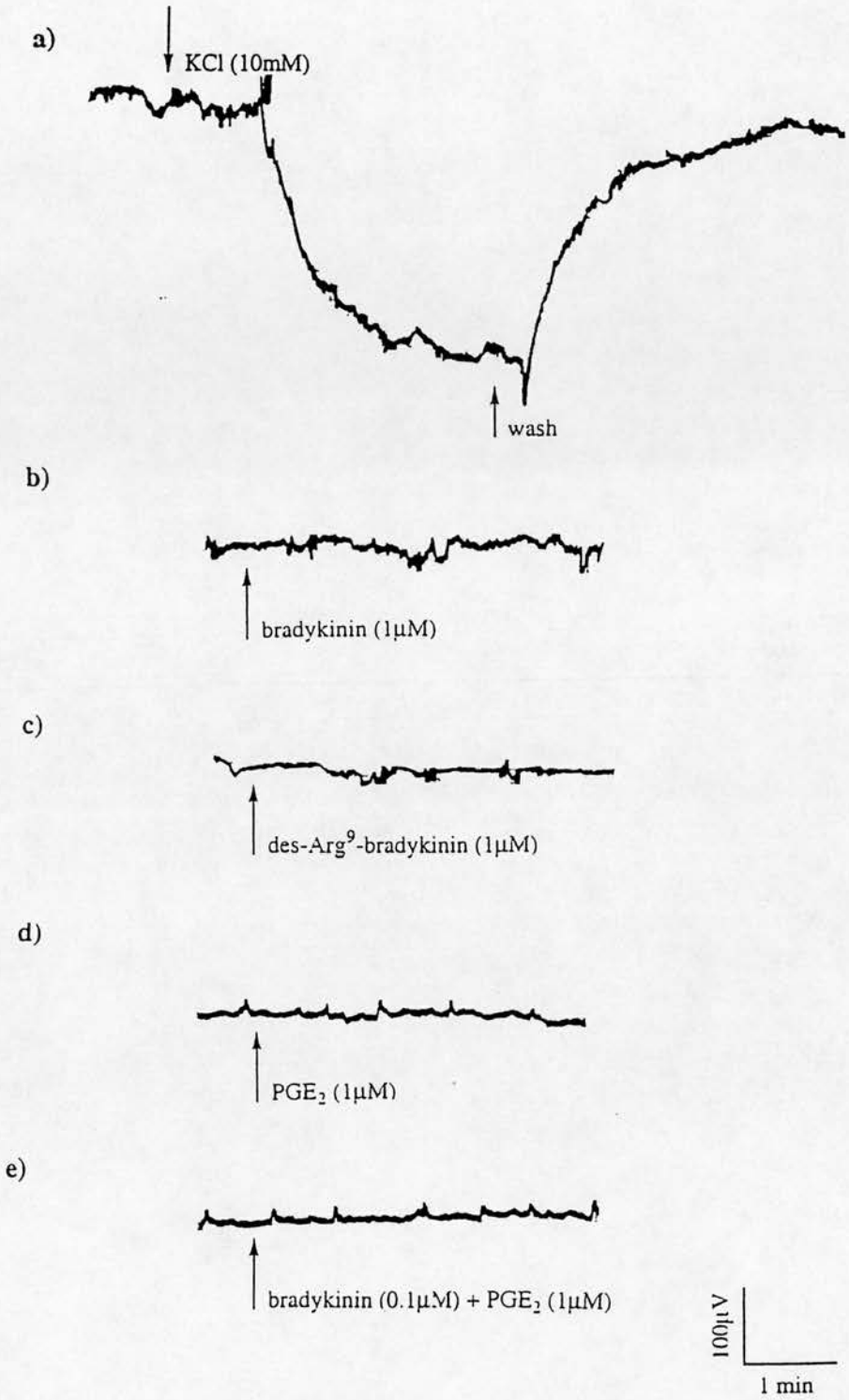



Figure 8.1 Typical chart records from the isolated rat vagus illustrating the depolarisation to (a) KCl, and the lack of effect on membrane potential of (b) bradykinin, (c) des-Arg$^9$-bradykinin, (d) $PGE_2$ and (e) a combination of bradykinin and $PGE_2$. Similar responses were observed using other isolated nerves (tibial, tibialis, sciatic and saphenous). Downward deflections indicate depolarisation. The contact time of drugs was 3 - 5min.

## 8.4 DISCUSSION

The results of the current study, obtained using the 'grease gap' technique, have shown that, in none of the isolated nerves (vagus, tibial, tibialis, sciatic and saphenous) examined, did either of the peptides, BK or the $B_1$ receptor agonist, des-$Arg^9$-BK, cause any change in membrane potential; however, all the preparations were capable of responding, as they were depolarised by KCl. These results are in contrast with the findings of other studies, also using the 'grease gap' technique in rat isolated nerve preparations (vagus and superior cervical ganglion), which show that changes in membrane potential (depolarisation and/or hyperpolarisation) do occur to other peptides such as angiotensin (Hawcock et al., 1992) or to non peptides such as prostanoids (e.g. $PGI_2$, Birrell, 1990), 5-HT (Ireland, 1987; Ireland & Jordan, 1987; Ireland & Tyers, 1987) and ATP (Trezise et al., 1993).

It has been reported in the rabbit isolated aorta that contractile responses to BK or to des-$Arg^9$-BK increase in magnitude (over 3-6hr, via $B_1$ receptors) in a time-dependent manner (Bouthillier et al., 1987; Farmer et al., 1991a). In contrast, the results of the present investigation suggest that BK receptors were not induced because repetitive challenge with BK or des-$Arg^9$-BK over 4 - 5hr did not change the membrane potential of the isolated nerves.

Although in the current study, BK had no effect on the membrane potential of any of the isolated nerves studied, it is possible that an effect could be produced by the combination of BK with inflammatory agents such as $PGE_2$. Indeed, it has been

shown that although $PGE_2$ causes little or no increase in afferent neural discharge, it greatly potentiates the response to bradykinin (Grubb et al., 1991; Birrell et al., 1993). However, in the present study $PGE_2$, either alone or in combination with BK, failed to cause any change in membrane potential. The possibility that a change in membrane potential may be produced upon the combination of BK with other prostanoids (e.g. $PGI_2$) or with other inflammatory mediators (e.g. 5-HT or ATP) requires further investigation. In the *in-vitro* study by Marsh et al (1987), the C-fibre excitant, capsaicin, was found to depolarise electrically-stimulated vagal sensory C-fibres. In view of this study, it would be interesting to determine the actions of BK and its analogues in preparations which are electrically-stimulated.

The use of whole nerve segments may explain the lack of effect of BK in the present study, as it may be that BK receptors are only found on the terminals (nociceptors) of the nerve fibres. Thus, in order to observe effects to BK the nerve plus the innervating organ may be required. Indeed, there are many nerve-organ *in-vitro* preparations which show responses to BK such as the skin-saphenous (Chahl & Iggo, 1977; Lang et al., 1990), cornea-ciliary (Belmonte, 1994), testis-superior spermatic (Kumazawa & Mizumura, 1983) and trachea-vagus (Fox et al., 1993) nerve preparations. Radioligand binding studies would help establish whether or not BK receptors are present in segments of whole rat nerve. It is also possible that although BK receptors are present in rat nerves, they are not functional because they may not be linked to, for example, second messengers.

## 8.5 SUMMARY

In summary, no change in the membrane potential of isolated rat nerves (vagus, tibial, tibialis, sciatic and saphenous), studied using the 'grease-gap' technique, was observed to BK, alone or in combination with $PGE_2$, or to the $B_1$ receptor agonist, des-$Arg^9$-BK.

## *SECTION 9*

# *NEURAL AND NON-NEURAL ACTIONS OF BRADYKININ AND ITS ANALOGUES IN THE ELECTRICALLY-STIMULATED RAT VAS DEFERENS.*

## 9.1 INTRODUCTION

The exogenous application of the nonapeptide, bradykinin (BK), to preparations of smooth muscle *in-vitro*, has been studied extensively, where it causes contraction and / or relaxation, depending on the preparation and tissue used (Erdös, 1979; Taylor et al., 1989; Farmer & Burch, 1992). The responses to BK are mediated by BK receptors which have been classified into the $B_1$ and $B_2$ subtypes (Regoli & Barabé, 1980). The vast majority of the physiological and pharmacological effects of BK appear to be mediated via $B_2$ receptors (See Taylor et al., 1989; Bathon & Proud, 1991; Steranka & Burch, 1991; Bhoola et al., 1992; Burch & Kyle, 1992; Farmer & Burch, 1992).

Evidence that there may be different subtypes of the $B_2$ receptor has been provided by the present neuropharmacological experiments (see Section 7), and by various studies, such as those in rat myometrial membranes (Liebmann, 1991), rat uterus, guinea-pig ileum and trachea (Plevin & Owen, 1988), murine neuroblastoma cells (Braas et al., 1988) and, of particular relevance to the present investigation, the studies by Huidobro-Toro et al. (1986), Llona et al. (1987) and Rifo et al. (1987) in the electrically-stimulated rat vas deferens (RVD). Application of BK to the electrically-stimulated RVD enhances the magnitude of the electrically-driven twitches (pre-junctional or neurogenic response) and increases the basal tension of the muscle (post-junctional or musculotropic response). Rifo et al. (1987) showed that, in the field-stimulated RVD, the "first generation" competitive $B_2$ receptor antagonist, D-Arg-[Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$]-BK (NPC349) was more potent at blocking the BK

neurogenic response than the BK musculotropic response. It was then concluded that these differences in potency observed with NPC349 could be interpreted as evidence for the notion that the pre- and post-junctional $B_2$ receptors are of a heterogeneous population (Rifo et al., 1987).

All of the 'first generation' antagonists for the $B_2$ receptor are of weak affinity and potency ($pK_B$ = 5 - 7) in smooth muscle preparations (Burch et al., 1990). However, more recently, $B_2$ receptor antagonists have been synthesised and shown to have potencies two or three orders of magnitude greater than the earlier compounds (Hock et al., 1991; Kyle et al., 1991; Lembeck et al., 1991; Burch & Kyle, 1992). One such potent $B_2$ receptor antagonist is D-Arg-[Hyp$^3$, Thi$^5$, D-Tic$^7$, Oic$^8$]-BK (Hoe140). In the present electrophysiological recordings from articular C-fibres (see Section 7), Hoe140 caused a mixed profile of antagonism; there was surmountable antagonism of BK-induced sensitisation of mechanically-evoked discharge, whereas insurmountable antagonism of BK-induced excitation occurred. These differences in antagonism of Hoe140 can be taken as evidence to suggest the existence of subtypes of the $B_2$ receptor.

Since the neuropharmacological experiments studying the effects of BK and BK analogues on articular mechanonociceptors were performed *in-vivo* (see Section 7), difficulties can arise in the interpretation of results; the difficulties associated with *in-vivo* experimentation include the problems of clearly establishing the mechanisms of drug action, for example, because of the possible contribution of blood or tissue borne mediators. Therefore, it is desirable to investigate the actions of BK and BK

analogues (in particular Hoe140) using a nerve preparation, *in vitro*. One such *in vitro* nerve preparation was the 'grease gap' technique as described in Section 8. However, since this neural preparation had no effects to BK it was not used further. It was decided to use the *in-vitro* electrically-stimulated rat vas deferens, a preparation known to contain neuronal (albeit on sympathetic neurones) BK receptors (Llona et al., 1987), in order to investigate in detail the actions of BK and its analogues.

The aims of the present study in the electrically-stimulated RVD were threefold. Firstly, to characterise the BK receptors involved in the BK-induced neurogenic and musculotropic responses. Secondly, to determine the profile of antagonism on the pre- and post-junctional effects of BK using a range of BK receptor antagonists. Thirdly, to examine in detail the effects of Hoe140 on the BK-induced responses.

Two preliminary studies were also performed in the electrically-stimulated RVD. In the first study, the presynaptic neurotransmitter responsible for the basal electrically-induced twitches, and that for the BK-induced enhancement in the electrically-induced twitch was investigated. The second preliminary investigation examined the role of protein kinase C in the neurogenic and musculotropic responses to BK.

## 9.2 MATERIALS & METHODS

### 9.2.1 Preparation of isolated vas deferens

Adult male Wistar rats (200-350g) were killed by cervical dislocation and the vasa deferentia immediately removed. The surrounding connective and adipose tissue and the adjoining blood vessels were removed from the vas deferens. Only the prostatic segment (1-1.5cm) of the vas deferens was used in the experiments.

Each prostatic segment was immersed in a 5ml organ bath containing Krebs solution of the following composition (mM): NaCl 118, KCL 5.4, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_2$ 25, glucose 11.1 and $CaCl_2$ 2.5. The Krebs solution was gassed with 95% $O_2$ and 5% $CO_2$ and maintained at 35°C.

Each prostatic segment was suspended between two parallel platinum electrodes which were used for transmural electrical stimulation. Electrical pulses were delivered by a multi-stimulator (Digitimer System-D330) at a frequency of 0.33Hz, 1msec pulse duration and at supramaximal voltage. To record isometric muscular contractions, the segments were connected to a force transducer (Dynamometer UF1) coupled to a chart recorder (Lectromed MT8-PX).

Prior to electrical field-stimulation or the application of drugs, the tissues were allowed to equilibrate for 60min with 0.5g of basal tension. During this equilibration period, the tissues were washed with Krebs solution every 15min and the tension re-adjusted to 0.5g.

### 9.2.2 Agonist / antagonist protocols

Agonists were added to the field-stimulated vas deferens in a cumulative manner. After completion of a cumulative log concentration-response curve, field-stimulation of the tissues was stopped and the vas deferens washed with Krebs solution every 2min for 10min. A further 20min equilibration period (no electrical stimulation) was allowed before constructing the next cumulative concentration-response curve.

To evaluate the potency of the bradykinin (BK) antagonists, cumulative concentration-response curves to BK were constructed in the absence and presence of the test antagonist. Antagonists were added to the organ baths, in the absence of electrical stimulation, 20min prior to the construction of the BK concentration-response curves. Three successively increasing concentrations of antagonist were equilibrated with the vas deferens, with each concentration of antagonist being washed out before the addition of the next higher concentration. In another series of BK antagonist studies, BK cumulative concentration-response curves were constructed before and after co-incubation of NPC349 (30 and 100μM) at varying time periods with Hoe140 (30nM).

Antagonist washout experiments were performed by constructing cumulative concentration-response curves to BK before and after the addition of a high concentration of the BK antagonist. To investigate the specificity of the BK antagonists in the vas deferens, neurogenic and musculotropic response curves were

constructed to noradrenaline, angiotensin II and U46619 before and after the addition of the BK antagonists.

In preliminary studies, neurogenic and musculotropic cumulative concentration-response curves to BK, were constructed before and after prazosin, propranolol, atropine, mepyramine, ranitidine, thioperamide, ondansetron, GR113808, GR82334, Men10207, indomethacin and staurosporine (0.3-1μM). These various agents were also added (1μM) during basal electrically-evoked stimulation of the rat vas deferens.

In another series of preliminary experiments, the $P_2$ purinoceptor was desensitised by two successive additions of α,β-methylene-ATP (10μM). The effects on basal electrically-evoked twitches, and the effects of BK (1μM), ATP (1mM) and U46619 (1μM) were obtained before and after inducing desensitisation to α,β-methylene-ATP.

### 9.2.3 Data analysis

Increases in basal muscle tension (musculotropic response) are expressed as a percentage of the maximal response that was attained by the test agent. The potentiations in the magnitude of the electrically-driven twitches (neurogenic response) are quantified as the percentage of the maximal response above the basal twitch attained by the test agent. The potency of BK and the $B_2$ receptor agonists at producing the neurogenic and musculotropic responses are expressed as the

geometric mean (95% confidence limits) of the $EC_{50}$ (concentration which produces 50% of the maximal response) values.

In the BK antagonist studies, $pA_2$ and slope values were obtained from Schild plots using a curve fitting program (Baspak, Glaxo Research & Development). Incubation with Hoe140 decreased the maximal BK musculotropic response (insurmountable antagonism) such that conventional Schild analysis could not be used to determine the potency of the antagonist. Nevertheless, an apparent $pK_B$ for Hoe140 was derived using a double regression plot (Kenakin, 1984). Essentially, such a plot involves plotting 1/A vs 1/A' where A and A' are the equieffective concentrations of agonist in the absence and presence of Hoe140, respectively. An estimated $pK_B$ is then derived by using the gradient (G) of this plot in the Gaddum Equation ($pK_B = -\log ([B] / G-1)$, where B is the antagonist concentration).

### 9.2.4 Statistical analysis

Experimental values are given as the mean ± s.e.mean. *n* represents the number of vas deferens used. For the BK agonist studies, the Student's paired t-test was used to determine statistical differences between the neurogenic and musculotropic $EC_{50}$ values of each test agent. The un-paired t-test was used to determine statistical relevance between BK and $B_2$ receptor agonists at the neurogenic and at the musculotropic responses. For the $B_2$ receptor antagonist studies, the paired t-test was used to statistically compare the $pA_2$ and slope values at the neurogenic response with

those obtained respectively at the musculotropic response. P values less than 0.05 were considered to be statistically significant.

## 9.3 RESULTS

### 9.3.1 Neurogenic and musculotropic effects of bradykinin

Cumulative additions of BK (0.001 - 3μM) to the electrically-stimulated RVD produces two distinct effects (typical chart record shown in Figure 9.1); a potentiation in the magnitude of the electrically-driven twitch (neurogenic response) and an increase in the basal tension of the muscle (musculotropic response). Both the BK-induced neurogenic and musculotropic cumulative concentration-response curves were highly reproducible for at least four consecutive cumulative curves (Figure 9.2). The $EC_{50}$ values (nM) and 95% confidence limits for the initial BK neurogenic and musculotropic curves ($n$=14) were 109.3 (32.5 - 340.5) and 93.4 (28.5 - 345.5), respectively. These values were not significantly different ($P>0.05$). $EC_{50}$ values for the BK-induced neurogenic and musculotropic effects did not differ significantly ($P>0.05$) from their respective $EC_{50}$ values over three further BK cumulative concentration-response curves.

### 9.3.2 $B_1$ receptor studies

The bradykinin $B_1$ receptor agonist, des-Arg$^9$-BK (1 - 3000nM), had no effect on either the basal electrically-induced twitch response or on the basal muscle tension of the vas deferens. Even when cumulative additions of des-Arg$^9$-BK (1 - 3000nM) were repeated up to three times, it still failed to produce either a neurogenic or musculotropic response. The bradykinin $B_1$ receptor antagonist, des-Arg$^9$-Leu$^8$-BK (3

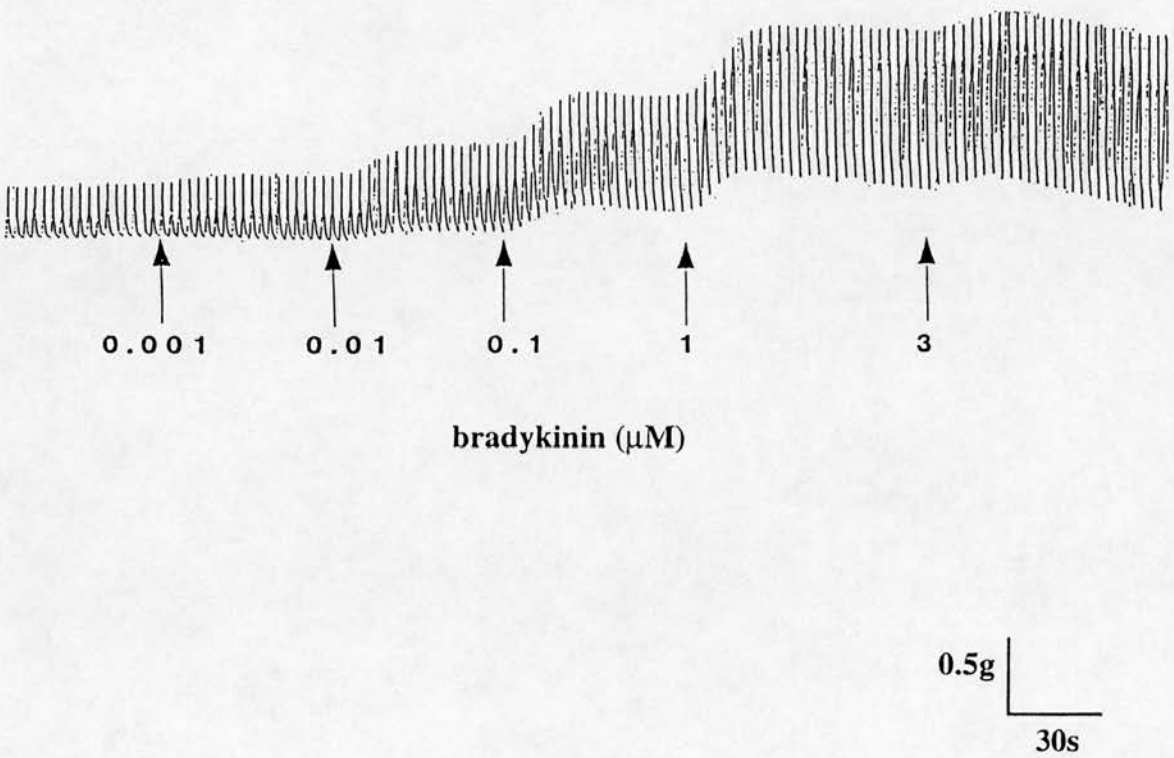



Figure 9.1 Typical chart record showing that cumulative additions of bradykinin to the electrically-stimulated rat vas deferens produces concentration-related increases in both the magnitude of the electrically-induced twitches (neurogenic response) and in the basal muscle tension (musculotropic response).

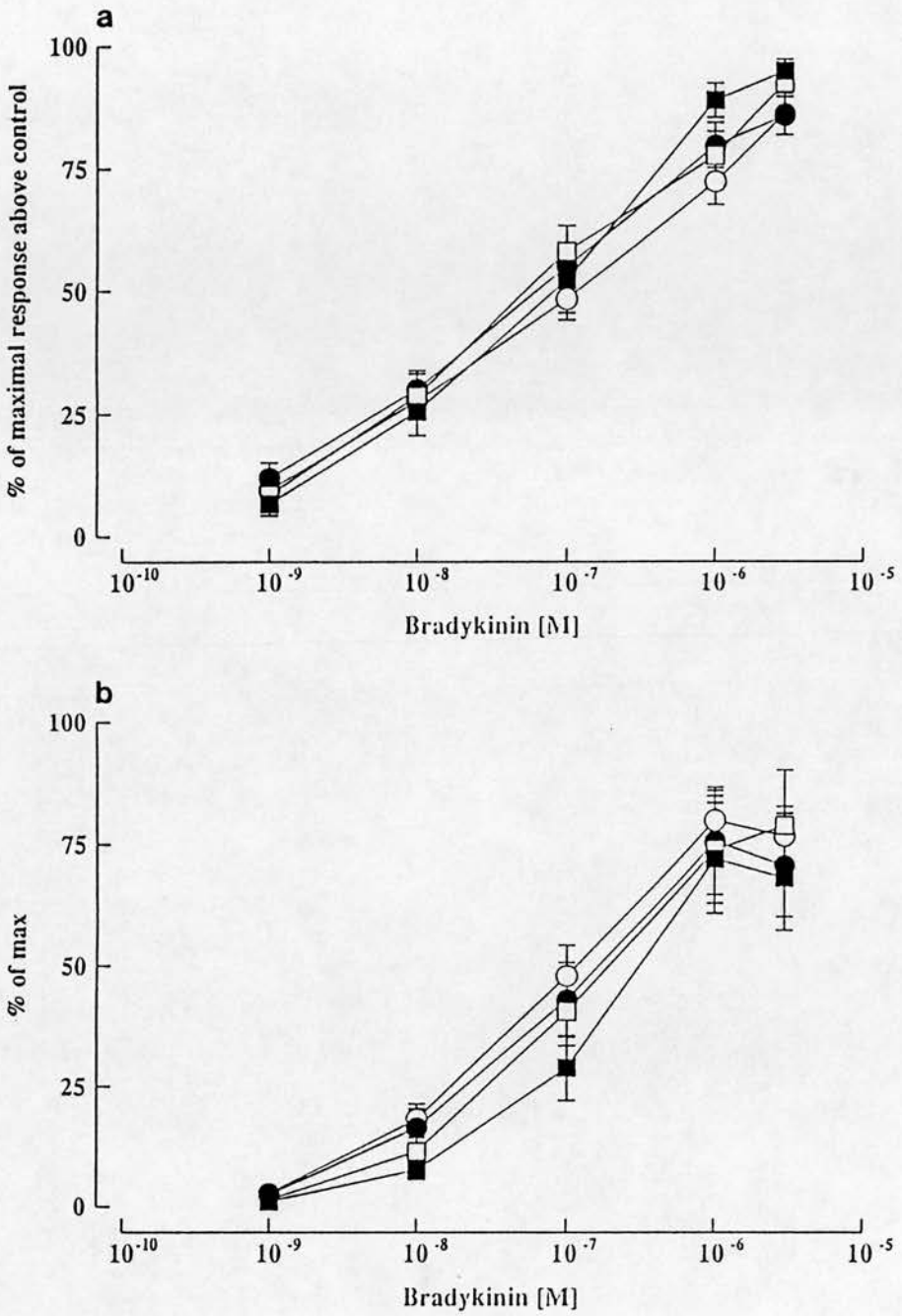



Figure 9.2 Reproducibility of cumulative log concentration bradykinin neurogenic (a) and musculotropic (b) response curves ( ○initial, ●second, □third and ■fourth curves). Each point is the mean ± s.e.mean (vertical bars) from eight experiments.

- 30μM), had no effect on either the neurogenic or musculotropic response curves to BK (Figure 9.3).

### 9.3.3 Effects of $B_2$ receptor agonists

Cumulative additions (1 - 3000nM) of BK and the $B_2$ agonists, Met-Lys-BK and Lys-BK, all produced concentration-related enhancements of the basal electrically-evoked twitch and the baseline muscle tension. Table 9.1 lists the $EC_{50}$ values with 95% confidence limits for the neurogenic and musculotropic responses of BK and two relatively selective $B_2$ agonists. As with BK, for each $B_2$ receptor agonist, the $EC_{50}$ for the neurogenic response did not differ significantly ($P>0.05$) from its respective $EC_{50}$ value for the musculotropic response. However, a significant difference ($P<0.05$) was found between agonists, with Met-Lys-BK being approximately 4.5-fold more potent at producing the neurogenic response than was BK (Table 9.1). The $EC_{50}$ values for the musculotropic responses induced by BK, Met-Lys-BK and Lys-BK were not significantly different from each other ($P>0.05$).

### 9.3.4 Effects of $B_2$ receptor antagonists on the bradykinin-induced neurogenic and musculotropic responses

All the $B_2$ receptor antagonists (Thi$^{5,8}$-DPhe$^7$-BK, adamanteacetyl-NPC349, DPhe$^7$-BK, NPC349, NPC567, Hyp$^2$-DPhe$^7$-BK, DArg-Hyp$^2$-DPhe$^7$-BK and Hoe140) tested produced concentration (0.01 - 100μM)-dependent rightward shifts of the cumulative neurogenic and musculotropic response curves to BK. All of the $B_2$ antagonists (1 - 100μM), except Hoe140, produced surmountable antagonism of the BK neurogenic

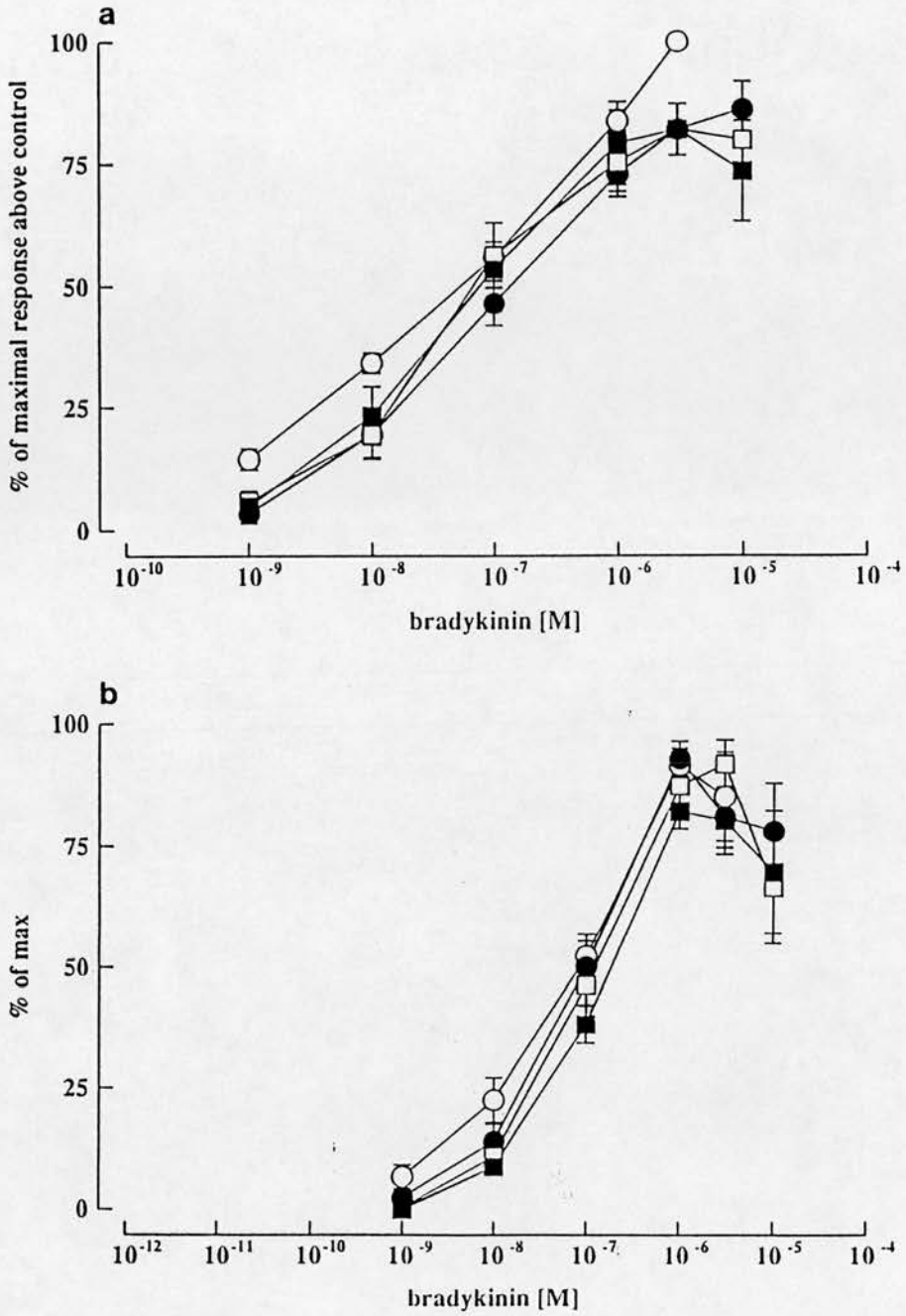



Figure 9.3 Effects of des-Arg$^9$-Leu$^8$-BK (●3μM, □10μM and ■30μM), incubated for 20min, on the control (○) bradykinin neurogenic (a) and musculotropic (b) cumulative log concentration-response curves. Each point is the mean ± s.e.mean (vertical bars) from eight experiments.

**Table 9.1** Potency of bradykinin (BK) and BK analogues at causing neurogenic and musculotropic responses in the rat vas deferens.

| *Peptide* | *n* | *Neurogenic response* $EC_{50}$ (nM) | | *Musculotropic response* $EC_{50}$ (nM) | *Musculotropic / Neurogenic ratio* |
|---|---|---|---|---|---|
| BK | 14 | 109.3 (32.5 - 340.5) | | 93.4 (28.5 - 345.5) | 0.9 |
| Met-Lys-BK | 8 | 23.6 (14.8 - 37.8) | * | 30.5 (17.2 - 54.1) | 1.3 |
| Lys-BK | 8 | 43.4 (14.6 - 128.9) | | 58.0 (29.1 - 115.5) | 1.3 |

Values are shown as the mean $EC_{50}$ (95% confidence limits). * indicates a statistically significant ($P<0.05$) difference compared to the BK neurogenic response

and musculotropic responses (typical example for NPC567 is shown in Figure 9.4). In contrast, Hoe140 (10-300nM), caused a concentration-related rightward shift and suppression of the maximal response of the BK musculotropic response curve, but surmountable antagonism of the BK neurogenic response curve (Figure 9.5).

Table 9.2 shows the $pA_2$ and slope values obtained with the various $B_2$ antagonists for both the BK neurogenic and musculotropic responses. Neither the $pA_2$ nor the slope values for the antagonists, DPhe$^7$-BK, Thi$^{5,8}$-DPhe$^7$-BK, D-Arg-Hyp$^2$-DPhe$^7$-BK, adamanteacetyl-NPC349, and NPC567 (1 - 100μM) at the BK neurogenic response differed significantly ($P>0.05$) from their respective values obtained at the BK musculotropic response. In contrast, Hyp$^2$-DPhe$^7$-BK (3 - 30μM) showed a modest, but significantly ($P<0.05$) greater potency for the BK musculotropic response ($pA_2 = 5.83 \pm 0.11$) than it did for the BK neurogenic response ($pA_2 = 5.54 \pm 0.12$). NPC349 (3 - 100μM) gave a significantly ($P<0.05$) higher slope against BK neurogenic responses (slope: $1.26 \pm 0.06$) than against BK musculotropic responses (slope: $0.99 \pm 0.03$). The slope of 1.26 obtained with NPC349 against BK neurogenic responses was significantly ($P<0.05$) greater than unity. The slopes of none of the other surmountable $B_2$ antagonists differed significantly ($P>0.05$) between those for the BK neurogenic and musculotropic responses, and neither did they differ significantly ($P>0.05$) from unity. Hoe140 (10 - 3000nM) was approximately 2 - 3 orders of magnitude more potent against both BK neurogenic and musculotropic responses than any of the other $B_2$ antagonists tested (Table 9.2).

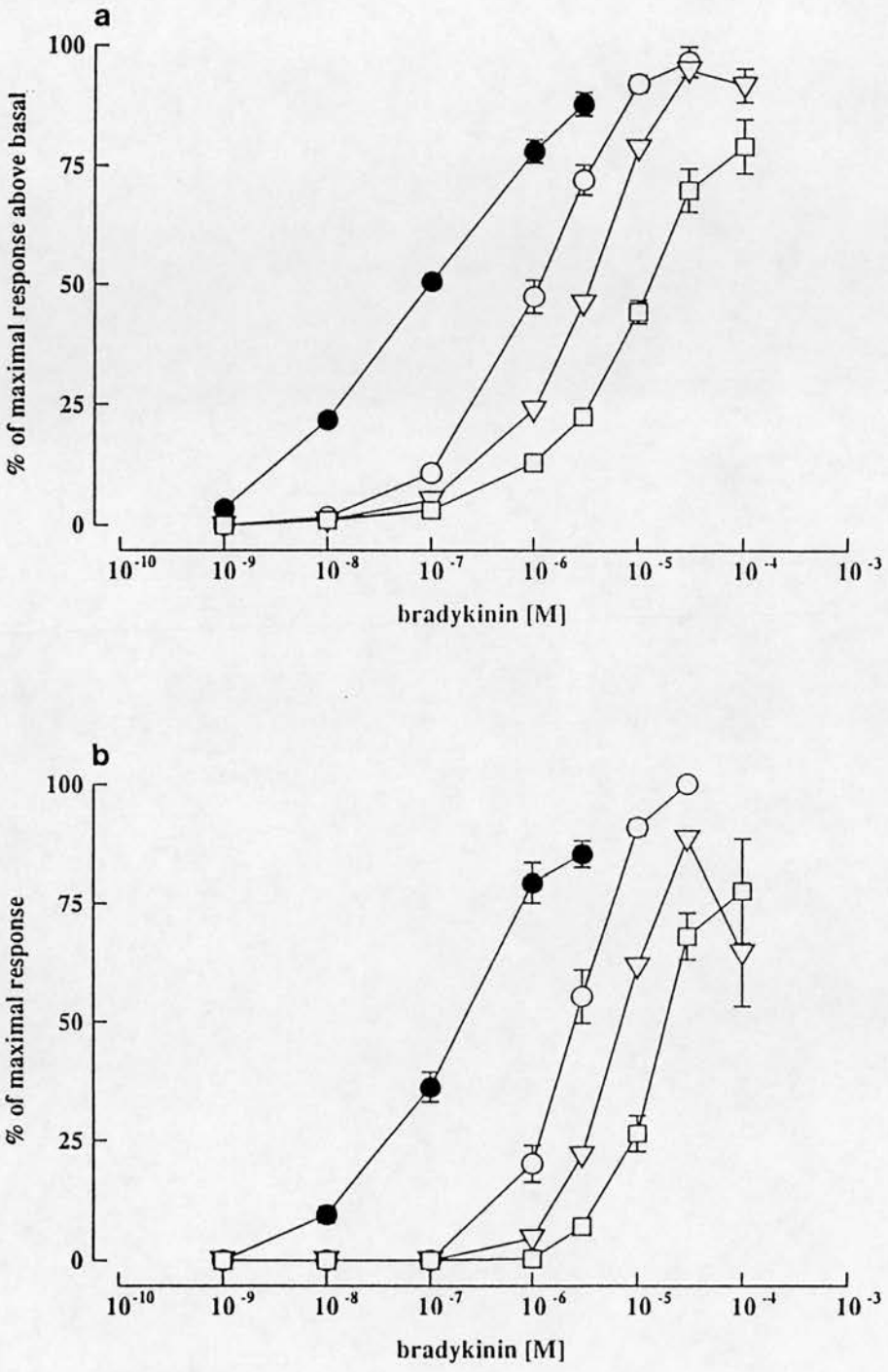



Figure 9.4 Effects of NPC567 ( ○10μM, ▽30μM and □100μM), incubated for 20min, on the control (●) bradykinin neurogenic (a) and musculotropic (b) cumulative log concentration-response curves. NPC567 = DArg-Hyp$^3$-DPhe$^7$-BK. Each point is the mean ± s.e.mean (vertical bars) from seven experiments.

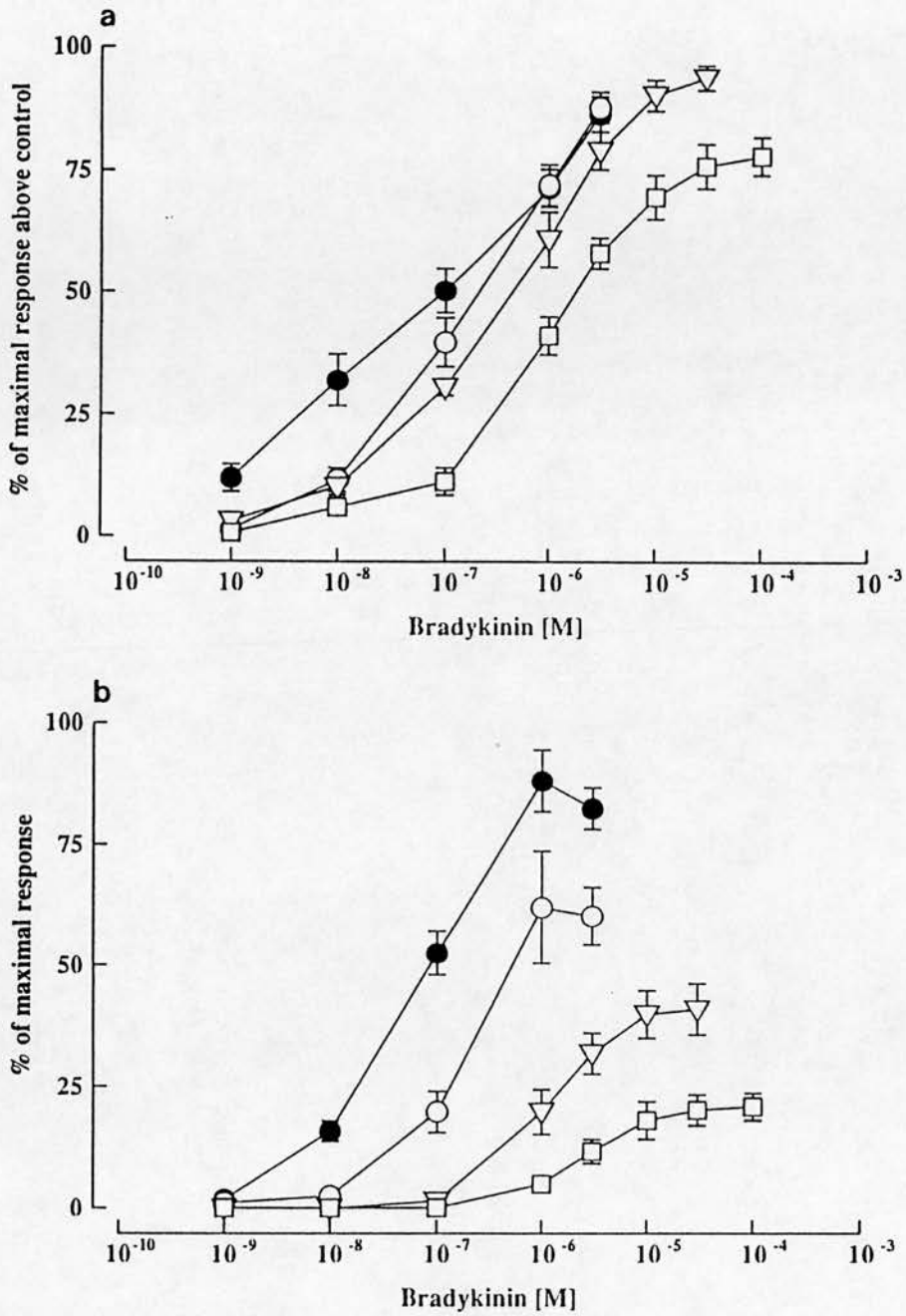



Figure 9.5 Effects of Hoe140 (○10nM, ▽30nM and □100nM), incubated for 20min, on the control (●) bradykinin neurogenic (a) and musculotropic (b) cumulative log concentration-response curves. Each point is the mean ± s.e.mean (vertical bars) from eight experiments.

**Table 9.2** The effects of $B_2$ receptor antagonists on the bradykinin neurogenic and musculotropic responses in the rat vas deferens.

| *Bradykinin (BK) antagonist* | | *neurogenic response* | | | *musculotropic response* | | |
|---|---|---|---|---|---|---|---|
| | *n* | $pA_2$ | | slope | $pA_2$ | | slope |
| NPC567 | 7 | 6.00 ± 0.17 | | 1.10 ± 0.09 | 6.29 ± 0.17 | | 0.90 ± 0.09 |
| NPC349 | 7 | 5.79 ± 0.07 | * | 1.26 ± 0.06 † | 6.31 ± 0.18 | * | 0.99 ± 0.03 † |
| $Thi^{5,8}$-$DPhe^7$-BK | 7 | 4.96 ± 0.49 | | 1.05 ± 0.21 | 5.57 ± 0.12 | | 0.96 ± 0.07 |
| $DPhe^7$-BK | 8 | 5.56 ± 0.06 | | 1.23 ± 0.08 | 5.45 ± 0.11 | | 1.19 ± 0.14 |
| $Hyp^2$-$DPhe^7$-BK | 7 | 5.54 ± 0.12 | * | 0.92 ± 0.10 | 5.83 ± 0.11 | * | 1.01 ± 0.10 |
| $DArg$-$Hyp^2$-$DPhe^7$-BK | 7 | 5.86 ± 0.19 | | 0.85 ± 0.08 | 6.00 ± 0.18 | | 1.15 ± 0.22 |
| Adamanteacetyl-NPC349 | 8 | 7.02 ± 0.25 | | 0.98 ± 0.15 | 6.94 ± 0.13 | | 1.14. ± 0.08 |
| Hoe140 | 8 | 8.50 ± 0.46 | | 1.19 ± 0.14 | # | | - |

# antagonist caused rightward shift and depression of the maximal response (apparent $pK_B = 9.01 \pm 0.15$ with 30nM Hoe140)
* $P<0.05$: denotes significant differences between the $pA_2$ values at the neurogenic and musculotropic responses
† $P<0.05$: denotes significant differences between the slope values at the neurogenic and musculotropic responses

None of the $B_2$ antagonists, in the concentration range tested (0.001-100μM), had any direct effect on the basal electrically-induced twitch response, nor did they affect baseline tension.

### 9.3.5 Effects of NPC349 on Hoe140-induced insurmountable antagonism of the bradykinin musculotropic response

Incubation (20min) with Hoe140 (30nM) caused a rightward shift and approximately 70% suppression of the maximal response of the BK musculotropic response curve (Figure 9.5). Increasing the incubation time of Hoe140 (30nM) from 2-180min had no effect on the rightward shift or on the suppression of the maximal response of the BK musculotropic curve (Figure 9.6). Incubation (20min) with the $B_2$ antagonist, NPC349 (3 - 100μM), produced surmountable concentration-dependent antagonism of the cumulative musculotropic response curve to BK (Figure 9.7 and Table 9.2). Co-incubation of NPC349 (30 and 100μM) for the final 18min of the Hoe140 (30nM) incubation period (20min), produced BK musculotropic response curves which were displaced upwards ('rectified') in a concentration-related manner, and shifted to the left (NPC349, 30μM) or to the right (NPC349, 100μM) as compared to the BK curve in the presence of Hoe140 (30nM) alone (Figure 9.8). In another related series of experiments, varying the incubation time (2 and 18min) of a single concentration of NPC349 (100μM) produced no differences in the subsequent BK musculotropic curves, where there were shifts to the right and upwards displacements of the curves as compared to the BK curve in the presence of Hoe140 alone (Figure 9.9).

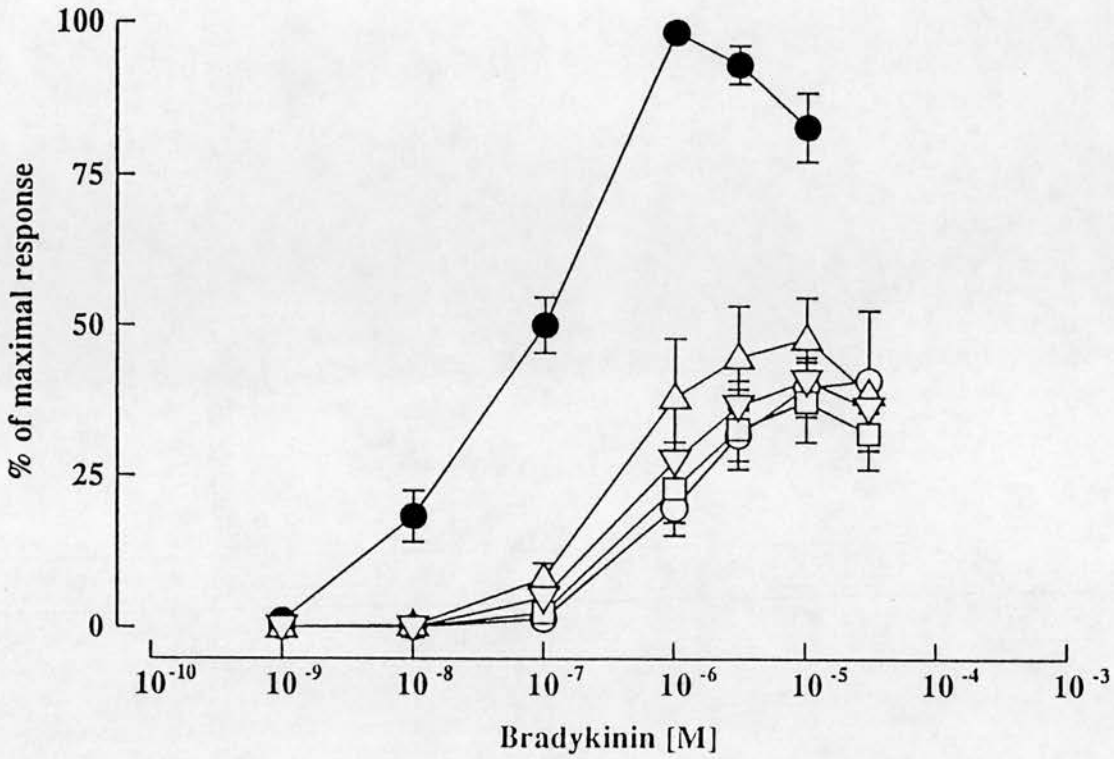



Figure 9.6 Effects of Hoe140 (30nM), incubated for 2 (○), 20 (□), 120 (△) and 180 (▽) min, on the bradykinin musculotropic log cumulative concentration-response curve (●). Each point is the mean ± s.e.mean (vertical bars) from eight experiments.

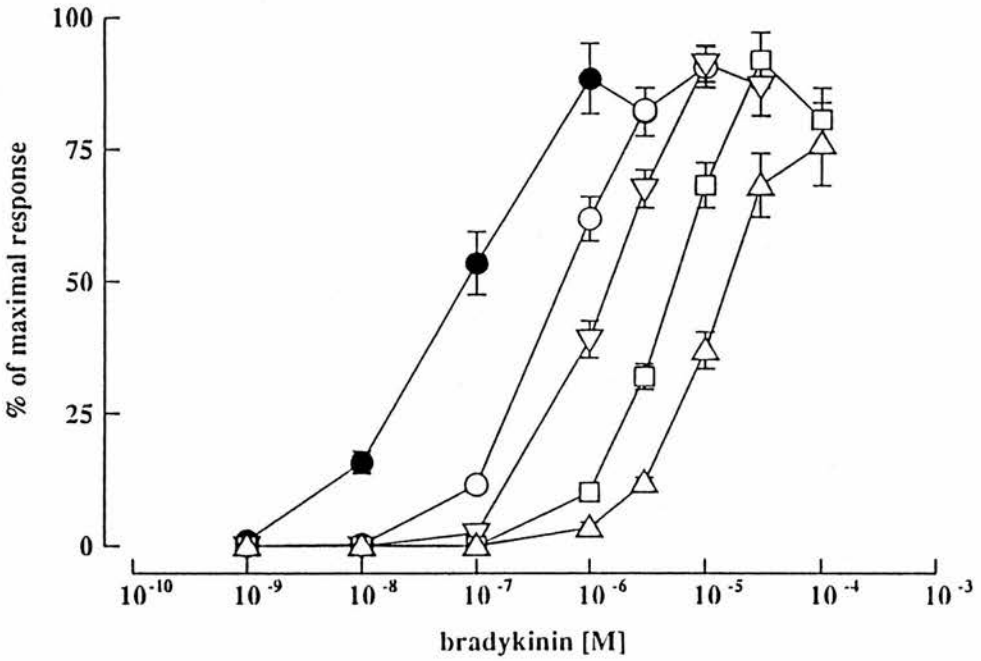



Figure 9.7 Effects of NPC349 ( ○3μM, ▽10μM, □30μM and △100μM), incubated for 20min, on the bradykinin musculotropic log cumulative concentration-response curve (●). Each point is the mean ± s.e.mean (vertical bars) from seven experiments.

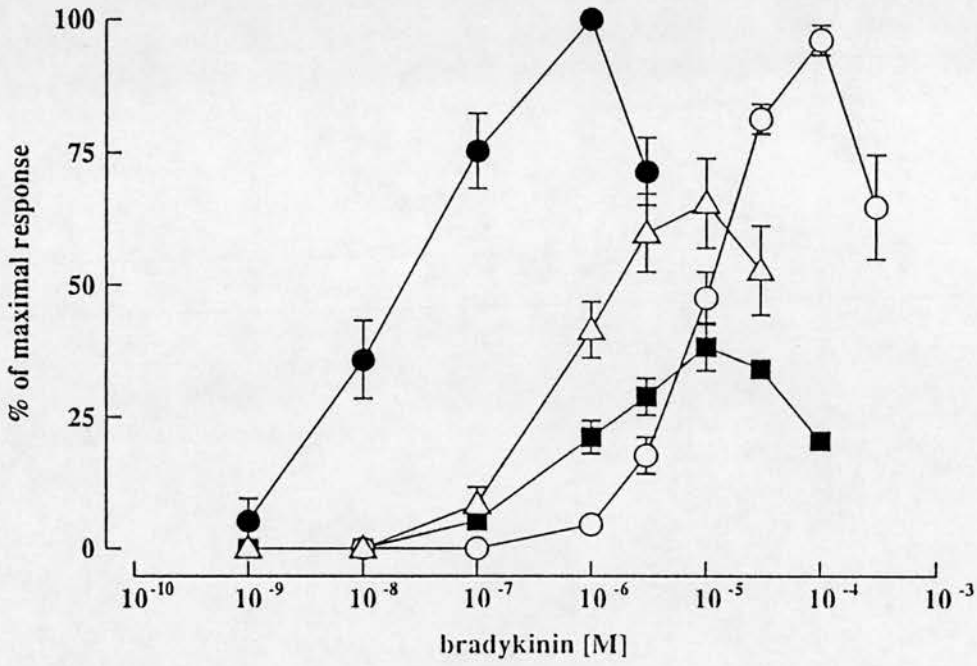



Figure 9.8 The effects of Hoe140 (30nM, 20min incubation) alone (■), or co-incubated with NPC349 (△30μM and ○100μM) for the final 18min of the 20min incubation period, on the control bradykinin musculotropic log cumulative concentration-response curve (●). Each point is the mean ± s.e.mean (vertical bars) from four to five experiments.

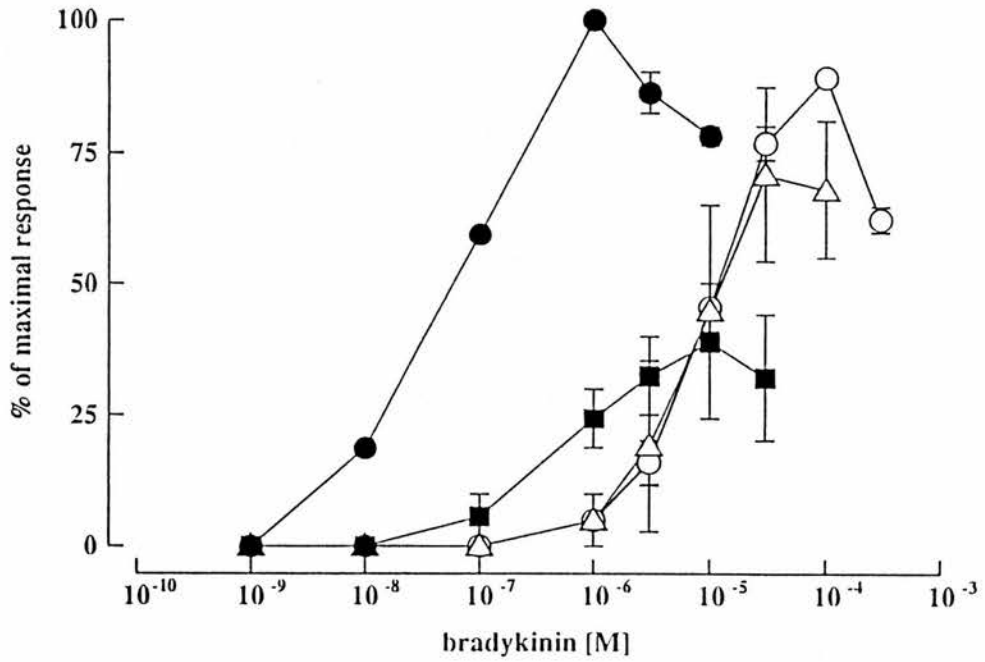



Figure 9.9 The effects of Hoe140 (30nM, 20min incubation) alone (■), or co-incubated with NPC349 (100μM) for the final 2 (△) or 18 (○)min of the 20min incubation period, on the control bradykinin musculotropic log cumulative concentration-response curve (●). Each point is the mean ± s.e.mean (vertical bars) from four to five experiments.

### 9.3.6 Specificity and wash-out experiments of $B_2$ receptor antagonists

The specificity of $B_2$ receptor antagonists (0.1 - 1μM) was tested against the neurogenic and musculotropic effects to noradrenaline, U46619 (thromboxane $A_2$ mimetic) and angiotensin II (0.01-100μM). Neither the neurogenic nor the musculotropic concentration-response curves obtained to any of these agonists were affected by any of the $B_2$ receptor antagonists examined in this study (n=3-4, data not shown). An example of such a specificity study for Hoe140 is illustrated in Figure 9.10 using an approximate $EC_{50}$ concentration of noradrenaline (1μM).

The effects of all the $B_2$ receptor antagonists (100nM, 20-180min incubation for Hoe140 and 100μM, 20min incubation for other antagonists) could be fully reversed on washing, since the BK-induced neurogenic and musculotropic response curves obtained after antagonist wash-out were similar to the initial curves without antagonist (n=4-8, data not shown).

### 9.3.7 Preliminary studies into the identity of the neurotransmitter in the electrically-stimulated rat vas deferens

Both the BK-induced neurogenic and musculotropic cumulative concentration-response curves (*n*=6-7) were unaffected by the following antagonists (1μM, 20min incubation): prazosin ($\alpha_1$ adrenoceptor, Figure 9.11), propranolol (non-selective β adrenoceptor), atropine (muscarinic cholinoceptor), 8-phenyltheophylline (non-selective adenosine receptor), mepyramine ($H_1$ receptor), ranitidine ($H_2$ receptor),

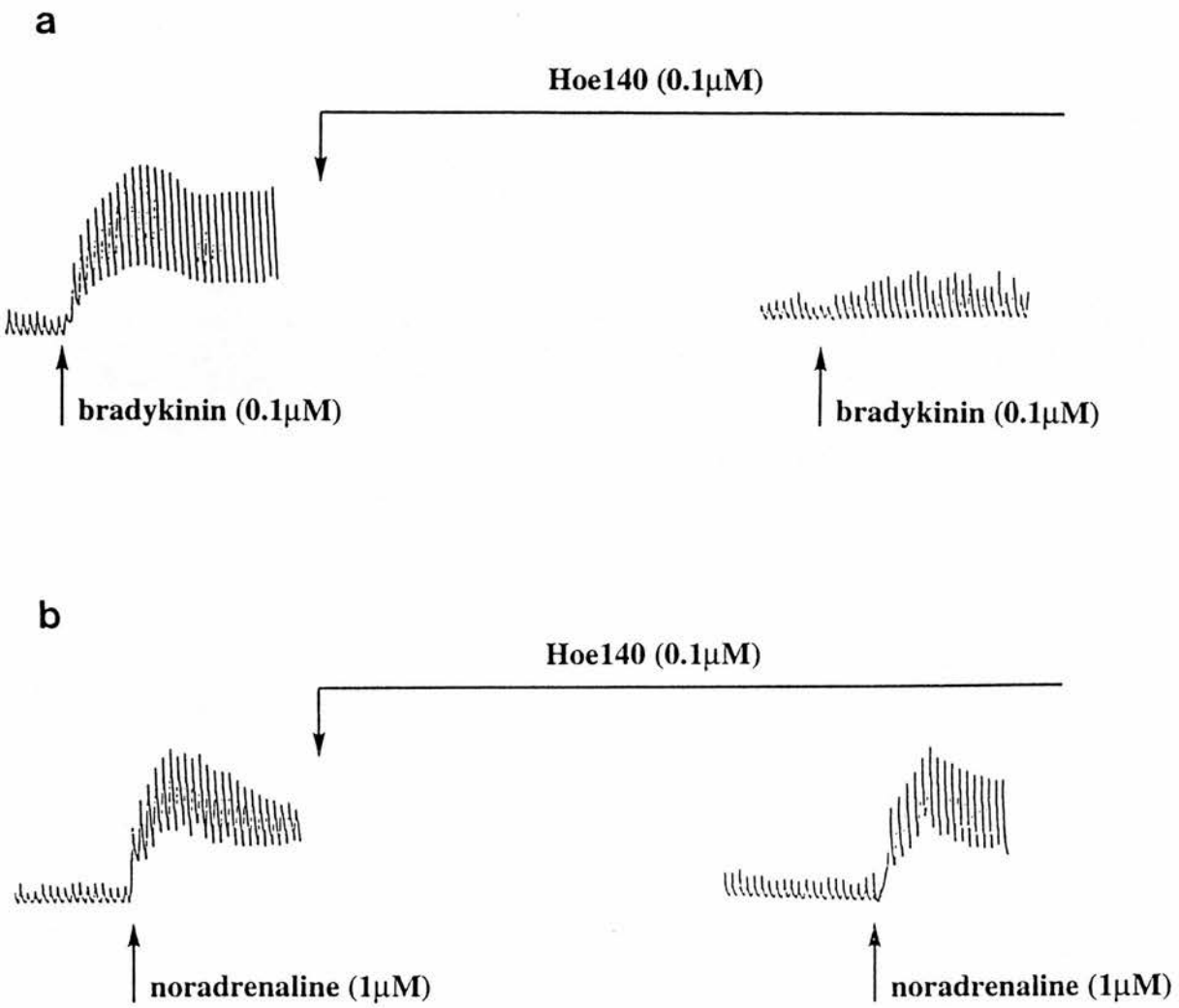



Figure 9.10 Chart records showing the effects of bradykinin (a) and noradrenaline (b) before and after incubation (20min) with Hoe140.

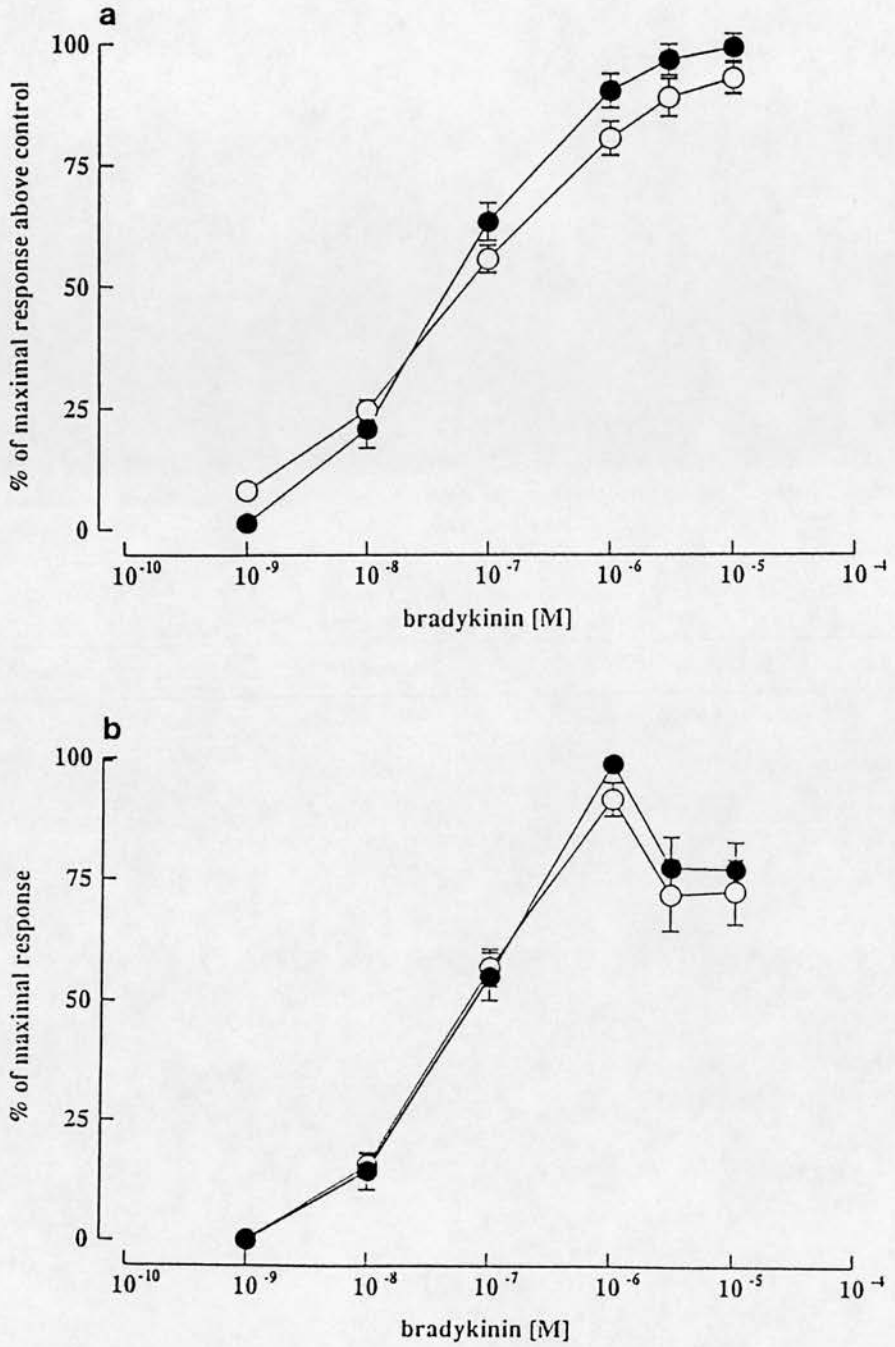



Figure 9.11 Effect of cumulative dosing with bradykinin on neurogenic (a) and musculotropic (b) responses of the rat vas deferens before (○) and after (●) incubating (20min) with prazosin (1μM). Each point is the mean ± s.e.mean (vertical bars) from seven experiments.

thioperamide ($H_3$ receptor), ondansetron (5-$HT_3$ receptor), GR113808 (5-$HT_4$ receptor), GR82334 ($NK_1$ receptor) and Men10207 ($NK_2$ receptor). The BK response curves were also unaffected in tissues incubated with the cyclo-oxygenase enzyme inhibitor, indomethacin (1μM) (Figure 9.12). None of these drugs had any effect on the basal electrically-induced twitches (1μM, *n*=6-7).

Basal electrically-evoked twitches, BK-induced enhancements in the magnitude of the electrically-evoked twitches, and the contraction induced by ATP, were reduced or abolished in preparations exposed to α,β-methylene-ATP (10μM) (Figure 9.13). The contraction produced by U46619 (1μM) was unaffected in preparations exposed to α,β-methylene-ATP (10μM) (Figure 9.13).

### 9.3.8 Preliminary second messenger studies in the electrically-stimulated rat vas deferens

The chart records in Figure 9.14 shows that the PKC inhibitor, staurosporine (1μM), preferentially blocked the BK musculotropic response (complete block) over the BK neurogenic response (partial block) in an electrically-stimulated RVD. Figure 9.15 shows the pooled data for the effects of staurosporine (1μM) in the RVD; the BK neurogenic and musculotropic cumulative concentration-response curves were both shifted to the right (greater shift at the BK musculotropic response) with approximately 40% and 85% suppressions in the maximal response, respectively.

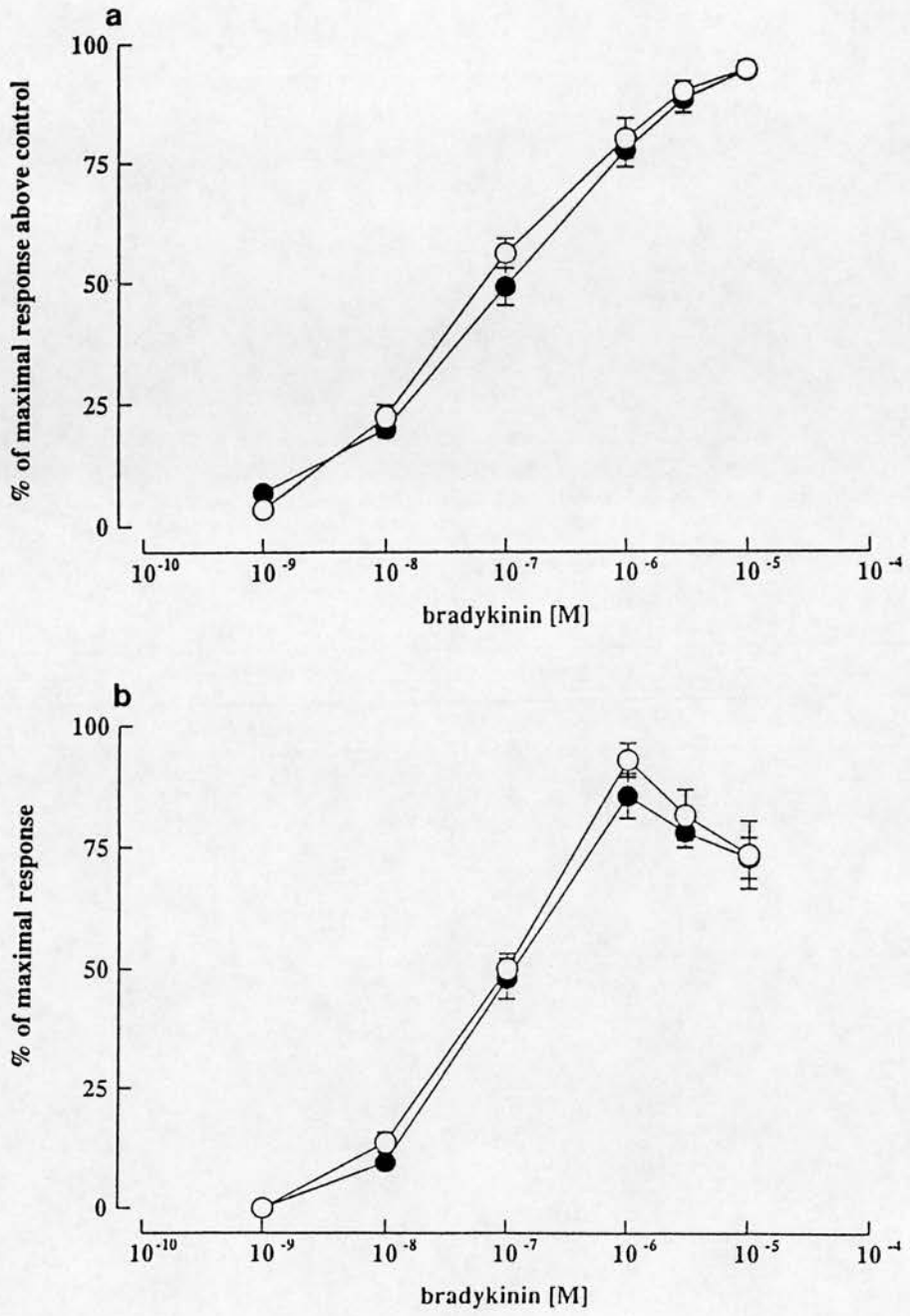



Figure 9.12 Bradykinin-induced neurogenic (a) and musculotropic (b) log cumulative concentration-response curves before (○) and after (●) incubating (20min) with indomethacin (1μM). Each point is the mean ± s.e.mean (vertical bars) from seven experiments.

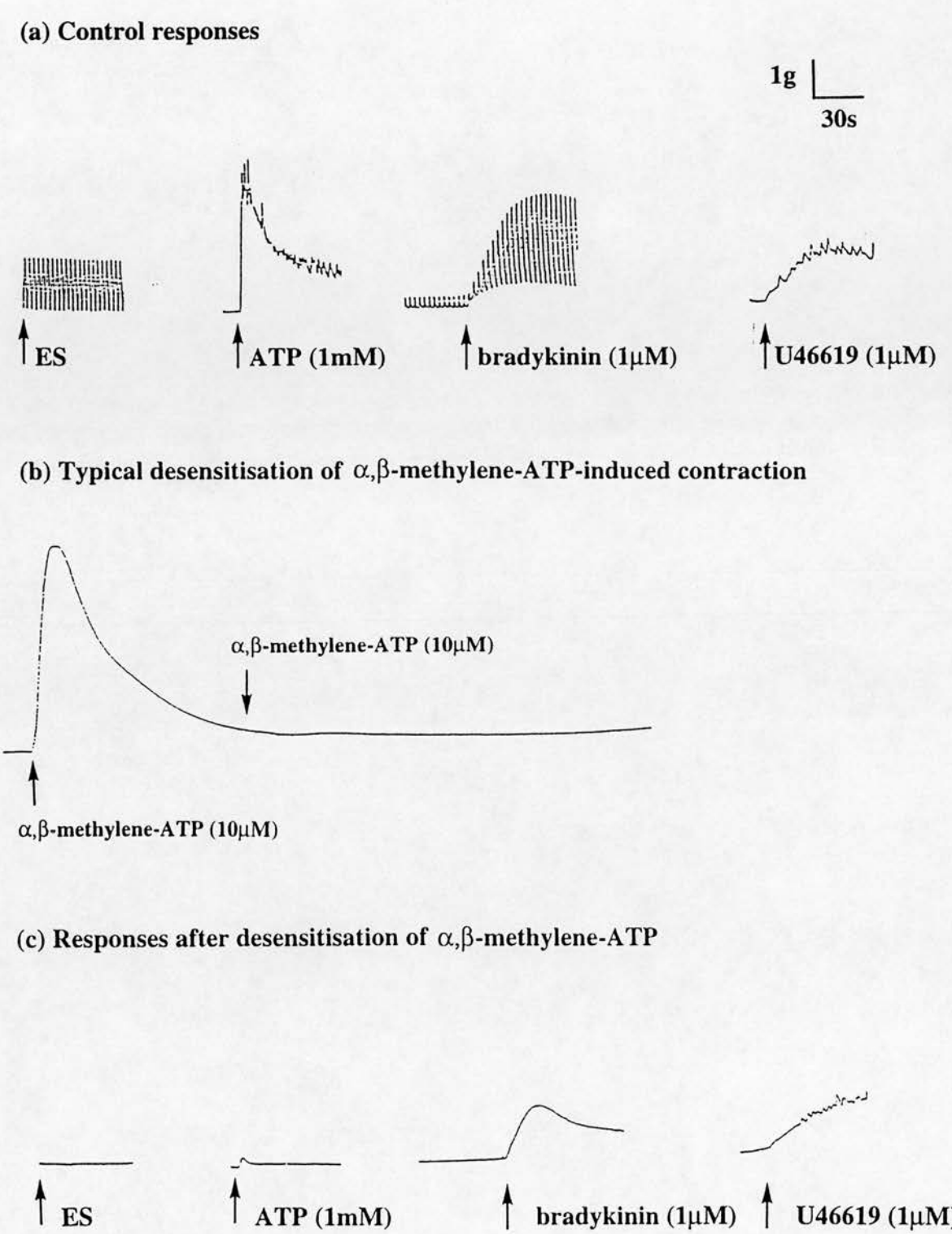



Figure 9.13 Chart records showing the effects of basal field electrical stimulation (ES), ATP (1mM), bradykinin (1μM) and U46619 (1μM) before (a) and after (c) desensitisation to α,β-methylene-ATP (10μM) (b).

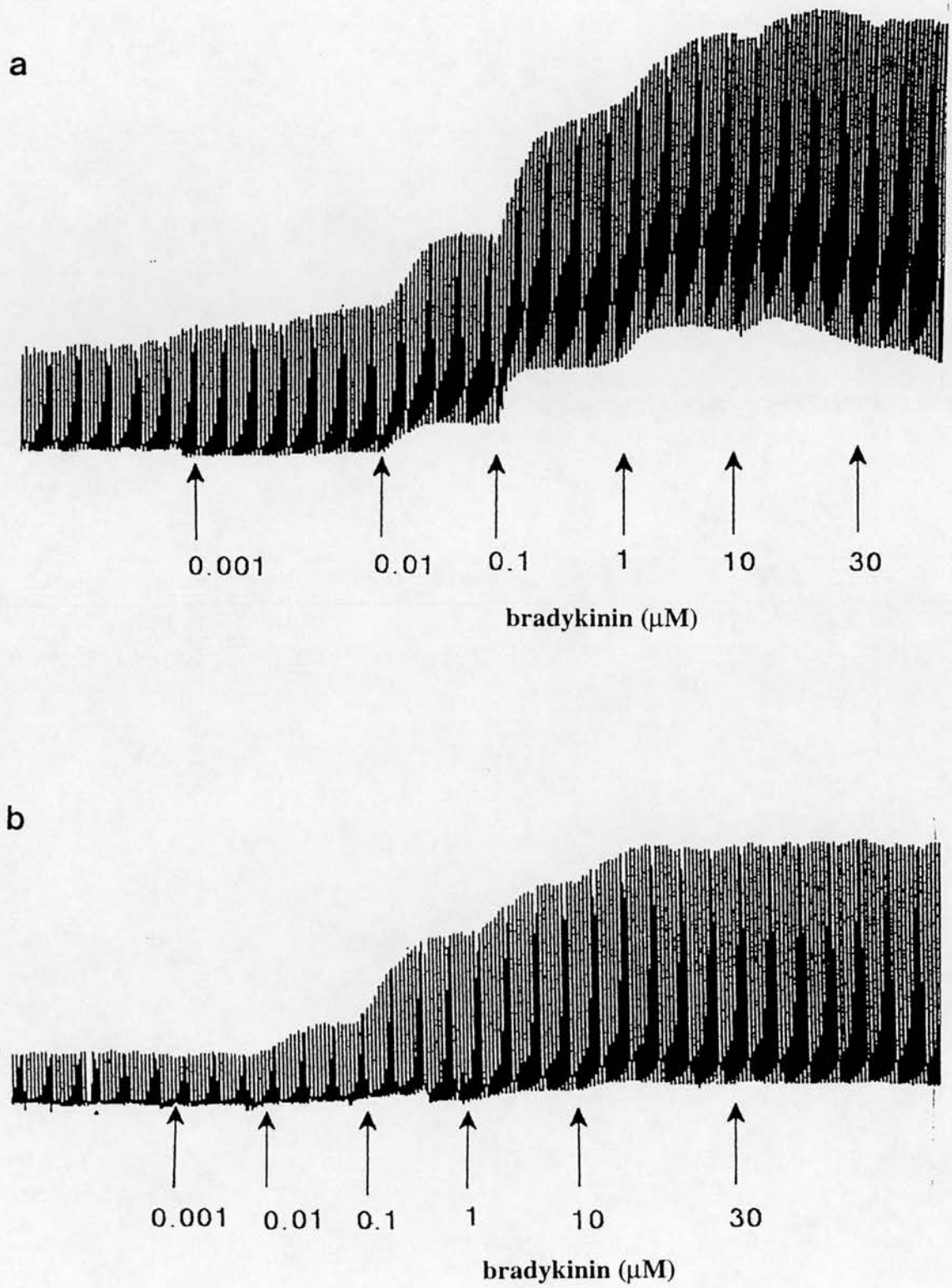



Figure 9.14 Chart records showing cumulative additions of bradykinin (0.001 - 30μM) before (a) and after (b) incubation (20min) with staurosporine (1μM). Note that in this preparation, staurosporine completely abolished BK-induced musculotropic responses but only partially blocked BK-induced neurogenic responses.

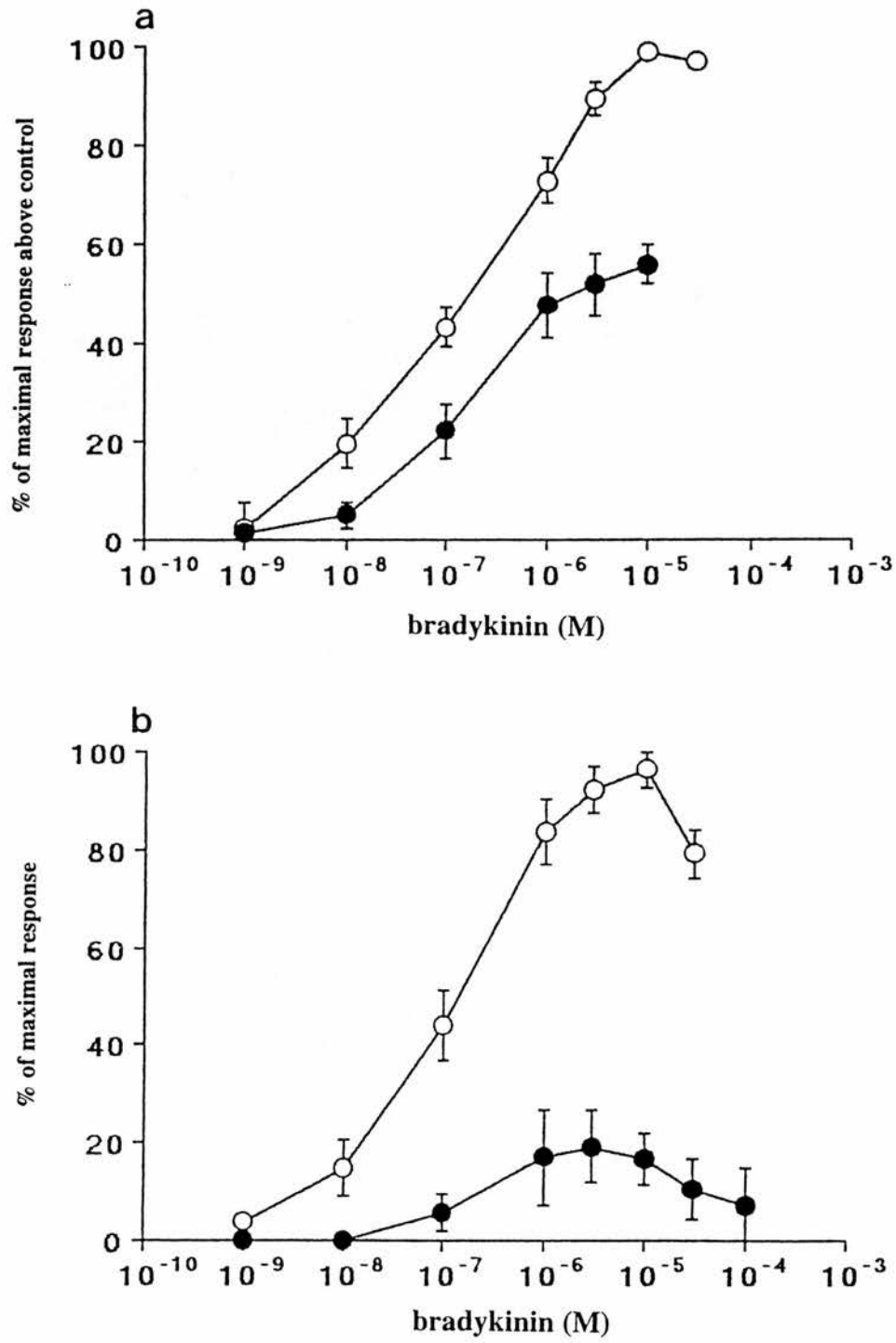



Figure 9.15 Bradykinin neurogenic (a) and musculotropic (b) log cumulative concentration-response curves before (○) and after (●) incubating (20min) with staurosporine (1μM). Each point is the mean ± s.e.mean (vertical bars) from four experiments

## 9.4 DISCUSSION

### 9.4.1 Bradykinin neurogenic and musculotropic responses

This study has shown that addition of BK to the electrically-stimulated RVD produces two responses; an enhancement in the electrically-driven basal twitches and an increase in the basal muscle tension. The former response has been shown to be mediated by neuronal pre-junctional BK receptors, and has been termed the BK neurogenic response, whereas the latter response has been shown to be mediated by post-junctional BK receptors located on smooth muscle and is referred to as the BK musculotropic response (Huidobro-Toro et al., 1986).

### 9.4.2 Mediators of the bradykinin neurogenic and musculotropic responses

The responses to BK in various preparations have been shown to involve the release of noradrenaline (Llona et al., 1991), histamine (Sharma, 1991), adenosine (Green et al., 1991), substance P (Lembeck & Holzer, 1979) and prostanoids (Juan et al., 1984). In contrast, the results of the present investigation have shown that the basal electrically-evoked twitches, and the BK-induced neurogenic response, does not involve $\alpha_1$, $\beta$, $H_1$, $H_2$, $H_3$, adenosine, 5-$HT_3$, 5-$HT_4$, $NK_1$ or $NK_2$ receptors nor the involvement of prostaglandins. Preliminary results using the $P_2$ purinoceptor tachyphylactic agent, $\alpha,\beta$-methylene-ATP (Meldrum & Burnstock, 1983), have shown that ATP is likely to be the neurotransmitter responsible for both the basal electrically-evoked twitches and the BK-induced enhancement in the magnitude of electrically-evoked twitches (Figure 9.13). In contrast, it has been reported in

biochemical studies by Llona et al. (1991) in the rat vas deferens, that noradrenaline was the presynaptic neurotransmitter that mediated the BK-induced enhancement of the electrically-induced twitches. However, in this study by Llona et al. (1991), high frequency field stimulation (three 10Hz trains) was used to induce the output of $^{3}$H-noradrenaline from the vas deferens after addition of BK, whereas in the present functional study, a much lower frequency stimulation (0.33Hz) was used. Therefore, it appears that, in the present study and in other similar studies (Huidobro-Toro et al., 1986; Llona et al., 1987; Rifo et al., 1987), the neurotransmitter released pre-junctionally in the field-stimulated RVD at low stimulation frequencies in response to BK (neurogenic response) is ATP rather than noradrenaline. Furthermore, since α,β-methylene-ATP-induced desensitisation is a recognised identification criterion for the $P_{2x}$ purinoceptor subtype (Burnstock & Kennedy, 1985; O' Conner, 1992), it can be further deduced that in the electrically-stimulated RVD, ATP acts on post-junctional $P_{2x}$ purinoceptors. In the light of an extensive literature in the vas deferens (for review see Campbell, 1987) which shows that ATP and noradrenaline are involved in co-transmission, it would be of interest to investigate the contribution made by these neurotransmitters, in response to BK, at different parameters of field-stimulation.

Since the BK-induced musculotropic response was not affected by a variety of receptor antagonists (prazosin, propranolol, atropine, 8-phenyltheophylline, mepyramine, ranitidine, thioperamide, ondansetron, GR113808, GR82334 and Men10207) or by indomethacin, it is likely that the post-junctional action of BK in the

RVD is mediated directly, and does not involve the release of other mediators as was observed to be the case for the BK-induced neurogenic response.

### 9.4.3 Bradykinin-induced neurogenic and musculotropic responses: preliminary second messenger studies

The protein kinase C (PKC) inhibitor, staurosporine, was found to inhibit both the BK-induced neurogenic and musculotropic responses. These results, therefore, implicate a role for PKC in the pre-and post-junctional transduction pathways activated by BK receptor stimulation. In agreement with the present study, involvement of PKC has been implicated in the transduction pathways activated by BK in various preparations, such as sensory neurones (Burgess et al., 1989), dorsal root ganglion neurones (Boland et al., 1991) and in the neonatal rat spinal cord-tail preparation (Dray et al., 1992). The present preliminary findings also indicate that PKC may not be the only second messenger mediator involved at the BK neurogenic and musculotropic responses because staurosporine only caused partial blocks of these BK effects. It has been shown that BK in various preparations can affect levels of inositol phosphates, arachidonic acid and cyclic AMP (for reviews see Taylor et al., 1989; Bathon & Proud, 1991; Farmer & Burch, 1992) and, therefore, it is possible that these mediators, alone or in combination, may play a role, along with PKC, in the transduction pathway of the BK-induced neurogenic and musculotropic responses. In the present investigation, staurosporine inhibited to a much larger extent the BK musculotropic effect than the BK neurogenic response (Figure 9.14 - 9.15). This differential sensitivity to staurosporine indicates that PKC is likely to be more important at mediating BK responses in muscle tissue than in neural tissue.

### 9.4.4 Bradykinin cumulative concentration-response curves

In the present study, BK was added in a cumulative manner. Preliminary experiments (data not shown) demonstrated that the BK neurogenic and musculotropic cumulative concentration-response curves were similar to their respective curves obtained by single additions of BK. The method of cumulative additions of BK has advantages over single concentration additions, as used by other investigators in the vas deferens (Huidobro-Toro et al., 1986; Rifo et al., 1987; Tousignant et al., 1987), in terms of the speed of construction of the concentration-response curves (<5min). Furthermore, by using cumulative additions of BK, it was possible to obtain three further cumulative concentration-response curves which were not significantly different from the first curve. As a result of this reproducibility, advantages were gained in the protocols for the antagonist experiments, in that it was possible to add three increasing concentrations of BK antagonist between cumulative curves (all antagonists used could be washed out), thus allowing easier and more accurate determination of antagonist potency.

The increases in the neurogenic and musculotropic responses to BK were generally similar to those observed by Rifo et al. (1987), who also used the electrically-stimulated RVD. However, in the study by Rifo et al. (1987), BK was 3-fold more potent at enhancing the neurogenic response than at increasing the musculotropic response, whereas in the present studies, BK was equipotent at both responses. A difference in strain of rat and in the segments of vas deferens used could explain the

different potencies observed with BK between the two studies. In the present investigation, Wistar rats were used, whereas Rifo et al. (1987) used Sprague-Dawley rats. Furthermore, Rifo et al. (1987) used the prostatic segment to determine the BK neurogenic response and the epididymal segment to determine the BK musculotropic response (neurogenic response greater at prostatic end, whereas musculotropic response greater at epididymal end), whereas in the present study the prostatic segment was used to determine both responses (similar tension changes for neurogenic and musculotropic responses). The prostatic segment from the Wistar rat is, therefore, a more suitable tissue on which to investigate comparisons between neurogenic and musculotropic responses.

### 9.4.5 $B_1$ receptors in the electrically-stimulated rat vas deferens

Classically, BK receptors are classified into the $B_1$ and $B_2$ subtypes (Regoli and Barabé, 1980). In the current investigation, the population of BK receptors in the electrically-stimulated RVD were not of the $B_1$ subtype, since the $B_1$ receptor agonist, des-Arg$^9$-BK, was inactive at producing either a neurogenic or a musculotropic response, and the $B_1$ receptor antagonist, des-Arg$^9$-Leu$^8$-BK, failed to antagonise neurogenic and musculotropic responses to BK. It has been shown that $B_1$ receptors can be induced, as shown by the increased responsiveness to des-Arg$^9$-BK, in the isolated rabbit aorta (Bouthillier et al., 1987). In contrast, as there was no response to des-Arg$^9$-BK (applied repeatedly with time) in the electrically-stimulated RVD, it is unlikely that $B_1$ receptors were induced in this preparation.

### 9.4.6 Effects of $B_2$ receptor agonists

BK, and the relatively selective $B_2$ receptor agonists, Met-Lys-BK and Lys-BK, all produced concentration-related enhancements of both neurogenic and musculotropic responses. No significant difference was found between the neurogenic and musculotropic $EC_{50}$ values for any of these agonists. This is in contrast to the study by Llona et al. (1987), which used whole vas deferens from Sprague-Dawley rats, where Met-Lys-BK and BK were more potent (26 and 3.5 fold, respectively) at enhancing the neurogenic response than at increasing the musculotropic response, although in agreement with the present study, Llona et al. (1987) demonstrated that Lys-BK was equipotent at both responses. Why such activity differences with Met-Lys-BK and BK arose between the two studies is not clear, but it may be attributable to segmental and/or species differences. However, such segmental or species differences do not explain why Lys-BK produced similar effects in the current study and that of Llona et al. (1987). In agreement with the findings of Llona et al. (1987), Met-Lys-BK was found to be more potent (approximately 4-fold) than BK at enhancing the neurogenic response, whereas no difference in activities was found between the agonists to increase baseline muscle tension (musculotropic effect). One hypothesis to explain these orders of activity observed with Met-Lys-BK, Lys-BK and BK is that the pre- and post-junctional $B_2$ receptors may not be of a homogeneous nature, and thus the data could suggest the existence of subtypes of the $B_2$ receptor in the RVD.

### 9.4.7 Effects of $B_2$ receptor antagonists

A range of $B_2$ receptor antagonists was used to determine their profile of antagonism for the BK neurogenic and musculotropic responses. All the $B_2$ receptor antagonists studied, with the exception of Hoe140 on the musculotropic response, caused concentration-dependent surmountable shifts of both the neurogenic and musculotropic concentration-response curves to BK (Table 9.2). The $B_2$ receptor antagonists, Thi$^{5,8}$-DPhe$^{7}$-BK and NPC349, had differing actions on the RVD between the present study using Wistar rats, and in the studies by Rifo et al. (1987) and Llona et al. (1987) using Sprague-Dawley rats. Whereas Thi$^{5,8}$-DPhe$^{7}$-BK was found to be a weak surmountable antagonist for both the BK-induced neurogenic and musculotropic responses in the present investigation, Llona et al. (1987) showed that Thi$^{5,8}$-DPhe$^{7}$-BK behaved as a competitive antagonist on the musculotropic response, but acted as a full agonist to enhance the basal twitch response. NPC349 was a more potent antagonist of the BK neurogenic response than of the BK musculotropic response in the study by Rifo et al (1987), whereas in the current investigation this antagonist behaved in the reverse manner (Table 9.2). These differences in the responses of Thi$^{5,8}$-DPhe$^{7}$-BK and NPC349 in the RVD of Wistar and Sprague-Dawley rats could reflect a heterogeneous population of the $B_2$ receptor between rat species.

The $B_2$ receptor antagonist, Hyp$^{2}$-DPhe$^{7}$-BK, gave an interesting result in the RVD in that, although it caused concentration-dependent competitive shifts of both the BK neurogenic and musculotropic response curves, it was more potent at antagonising the

BK musculotropic response than the BK neurogenic response. Although these potency differences observed with $Hyp^2$-DPhe$^7$-BK are not large, it could be taken as evidence to suggest that the pre- and post-junctional $B_2$ receptors are different.

The most interesting of the $B_2$ receptor antagonists studied in the electrically-stimulated RVD was Hoe 140, which was approximately 2 - 3 orders of magnitude more potent than any of the other $B_2$ receptor antagonists tested at both the BK-neurogenic and musculotropic responses (Table 9.2). A similar difference in potency between Hoe140 and other $B_2$ receptor antagonists has also been reported in a variety of other *in-vitro* smooth muscle preparations (Hock et al., 1991). The most striking difference between Hoe140 and the other $B_2$ receptor antagonists tested in the present study was in the differences in profile of antagonism observed between the BK neurogenic and musculotropic responses. Whereas all the other $B_2$ receptor antagonists studied showed competitive antagonism for both the BK neurogenic and musculotropic responses, Hoe140 displayed surmountable antagonism at the neurogenic response but insurmountable antagonism at the musculotropic response. Such insurmountable antagonism with Hoe140 has previously been shown in other preparations, such as the guinea-pig ileum (Griesbacher & Lembeck, 1992), rabbit jugular vein (Félétou et al., 1994), sheep femoral artery (Félétou et al., 1994) and in cultured colonic epithelial cells (Cuthbert et al., 1992). In the study by Cuthbert et al. (1992), the action of the BK analogue, Lys-BK, to raise intracellular calcium was blocked in an insurmountable fashion by Hoe140, although the action on electrogenic chloride secretion was competitively blocked.

### 9.4.8 Mechanisms of insurmountable antagonism of Hoe140 at the bradykinin musculotropic response

There are several mechanisms which could be responsible for the insurmountable action of Hoe140 on the BK-induced musculotropic response curve in the rat vas deferens. Hoe140 may act as 1) a 'non-specific' antagonist, 2) an irreversible antagonist, 3) a pseudo-irreversible antagonist, or 4) an allosteric antagonist, of BK. These various possible mechanisms are discussed in further detail below.

The possibility that the peptide, Hoe140, is a 'non-specific' antagonist such that it could block the chain of events that lead to the production of a response, or that it blocks a receptor unrelated to BK, is unlikely, because Hoe140 (0.1μM) was found not to affect the neurogenic and musculotropic responses induced by the peptide, angiotensin II, nor those by the non-peptides, noradrenaline and U46619. Thus, it is more likely that Hoe140 acts at the $B_2$ receptor, or at a site closely related to it.

Insurmountable antagonism can occur when covalent bonds are formed between the antagonist-receptor complex such that the number of receptors is reduced to an extent where it is no longer possible to obtain a full agonist response, and hence a suppression of the maximal response results. This explanation is unlikely to account for the insurmountable antagonism of Hoe140, since the antagonist could be washed out from the vas deferens following a 20-180min incubation period, and increasing the incubation time from 2 to 180min did not produce any change in the suppression of the maximal response (Figure 9.6). From these two results it is clear that Hoe140

does not form irreversible contacts with the BK receptor, and also that Hoe140 reaches equilibrium quickly (<2min). The finding that the suppression in the maximal response induced by Hoe140 could be reversed (Figure 9.8) by co-incubation with the $B_2$ competitive antagonist, NPC349, gives further evidence in favour of Hoe140 not being an irreversible antagonist, since if Hoe140 had bound in a covalent manner to the $B_2$ receptor, then no effect on the BK maximum would have of been expected if co-incubated with a competitive antagonist.

A more probable explanation to account for the insurmountable antagonism observed is that Hoe140 is acting as a slowly reversible (pseudo-irreversible) antagonist. The hypothesis of pseudo-irreversible antagonism is that receptor-bound antagonist dissociates so slowly such that the agonist cannot reach equilibrium with the antagonist-receptor complex within the time constraints of the experiment (problems of desensitisation, degradation of agonist etc.) and thus gives the apparent appearance that the antagonist has bound irreversibly (pseudo-irreversible antagonism). This hypothesis of pseudo-irreversible antagonism can be used to explain the results described above for the co-incubation experiments with Hoe140 and NPC349. Thus, Hoe 140 dissociates slowly from the $B_2$ receptor, but as it does, the vacated receptor becomes occupied by NPC349. The subsequent addition of BK then competes for receptors occupied by NPC349 as well as those occupied by Hoe140. On increasing the concentration of NPC349 a greater number of receptors become occupied by NPC349 than Hoe140. Since BK can surmount the antagonism by NPC349 (Figure 9.7), the maximal response attainable by BK increases in relation to the number of receptors occupied by NPC349. If it had been the case that Hoe140

did not dissociate from the receptor, then co-incubation with NPC349 would have had the effect of displacing further rightwards an already suppressed BK curve. Results similar to those in the present study, where reversal of the suppression in the maximal response, induced by an apparently insurmountable antagonist, by use of a competitive antagonist has been observed in the 5-HT (Bond et al., 1989) and angiotensin (Robertson et al., 1992) fields.

In order for this explanation of Hoe140 being a slowly-reversible antagonist, to be fully convincing, it is necessary for NPC349 to reach equilibrium with the $B_2$ receptor much more quickly than Hoe140. Although no experiments were conducted to determine whether NPC349 had reached equilibrium within the 20min incubation period, results from the study by Rifo et al (1987) in the vas deferens showed that using a lower incubation period of 5min a musculotropic $pA_2$ value of 6.4 is obtained for NPC349, which compares well with the $pA_2$ of 6.3 in the present study. Therefore, it is probably the case that NPC349 reaches equilibrium rapidly. However, since Hoe140 seems to reach equilibration within 2min (Figure 9.6) it would have to be postulated that for the explanations of the interactions of NPC349 and Hoe140 discussed above to be valid, NPC349 would have to reach equilibration even more rapidly, and certainly within 2min. Evidence that NPC349 had reached equilibration within 2min is provided in Figure 9.9, where a single concentration of NPC349 co-incubated for 2 or for 18min with Hoe140 produced similar rightwards shifts of the BK musculotropic curve.

Also for pseudo-irreversibility to explain the current findings, it would have been expected that shorter co-incubation times of NPC349 would have resulted in correspondingly greater suppression in the maximal response to BK, induced by Hoe140. Such incubation time-related reversal of Hoe140-induced response suppression with NPC349 could then be used to give an indication of the slow rate of dissociation of Hoe140 from the $B_2$ receptor. However, incubation of NPC349 for 2min or 18min produced similar reversals in the suppression of the maximal response induced by Hoe140 (Figure 9.9) suggesting that Hoe140 dissociates very rapidly from the BK receptor. Further evidence which suggests that the mechanism of action of Hoe140 may not be fully attributed to it being a slowly reversible antagonist, is the observation that since only a 2min incubation with NPC349 is required to reverse the suppression seen with Hoe140, then it becomes unclear as to why BK itself cannot reverse this suppression, assuming that BK, Hoe140 and NPC349 all act at the $B_2$ receptor. In conclusion, the data from the Hoe140 / NPC349 interaction experiments provide evidence (Figure 9.8) for Hoe140 being a pseudo-irreversible antagonist at the musculotropic $B_2$ receptor, although this evidence is not fully supported by the NPC349 time course experiments (Figure 9.9).

Allosteric modulation of the $B_2$ receptor by Hoe140 may be another explanation of why this antagonist operates to cause insurmountable antagonism of the BK musculotropic response. Essentially, allosteric antagonists act at a site close to (allosteric effector site), but not at, the agonist binding site, and have the action of diminishing (by changes in receptor affinity) the capability of the agonist-receptor complex to generate a response. Thus, in relation to the present results, it can be

hypothesised that Hoe140 causes insurmountable antagonism of the BK musculotropic response by acting at an allosteric effector site which then produces a change in the state of the BK receptor such that the action of BK becomes impaired. Furthermore, it can be postulated that NPC349 can also act at the allosteric site to reverse the effects of Hoe140. Therefore allosteric modulation of the $B_2$ receptor can be used, as an alternative to pseudo-irreversible antagonism, to explain the Hoe140 / NPC349 interaction experiments described above. From the present functional studies it is not possible to determine whether Hoe140 behaves as an allosteric antagonist or not. Studies using radioligand binding in the vas deferens, in order to determine whether BK binding to the $B_2$ receptor is affected by Hoe140, would aid in determining an allosteric modulatory role for Hoe140. Allosteric modulation has been proposed by Kaumann & Frenken (1985) to explain the insurmountable action of methysergide on the 5-HT vasoconstrictor responses in calf coronary arteries.

An alternative to the above possible mechanisms to explain the insurmountable nature of Hoe140 may be simply that Hoe140 acts on subtypes of the $B_2$ receptor, or on other types of BK receptor. The existence of subtypes of the $B_2$ receptor have been suggested in various preparations (e.g. in rat myometrial membranes, Liebmann et al., 1991; mouse neuroblastoma cells, Braas et al., 1988). In view of studies reporting the existence of novel types of BK receptor ($B_3$, $B_4$ and $B_5$ types: Farmer et al., 1989; Saha et al., 1990; Farmer et al., 1991c; Saha et al., 1991; Farmer & DeSiato, 1994) it is also conceivable that Hoe140 produces differing profiles of

antagonism at the pre- and post-junctional BK responses in the vas deferens by acting on different types of the BK receptor.

## 9.5 SUMMARY

In summary, the bradykinin receptors mediating bradykinin-induced neurogenic and musculotropic responses in the electrically-stimulated rat vas deferens are of the $B_2$ subtype - $B_1$ receptor agonists and antagonists were without effects. The differing profiles of action observed for some bradykinin $B_2$ receptor agonists and antagonists at the neurogenic and musculotropic responses (particularly Hoe140) may suggest that the $B_2$ receptors located pre- and post-junctionally in the rat vas deferens are not of a homogeneous nature.

Preliminary experiments have shown that the pre-junctional neurotransmitter mediating the basal electrically-induced twitches, and the bradykinin-induced neurogenic response, is likely to be ATP. Pilot experiments have provided evidence that protein kinase C is involved in the signal-transduction pathway for both the bradykinin-induced musculotropic (predominantly) and neurogenic responses, although the additional involvement of other second messengers cannot be ruled out.

*SECTION 10*

# *CONCLUDING DISCUSSION*

## CONCLUDING DISCUSSION

The present study has investigated the role of various mediators (purines, catecholamines and bradykinin) and their receptors in modulating C-fibre afferent mechanonociceptor discharge (spontaneous and mechanically-evoked) in normal rat ankle joints and in those with a chronic unilateral adjuvant-induced arthritis. The results from these investigations provide some insight into the relative importance of these mediators in contributing to the sensitisation of nociceptors that is associated with arthritis.

### 10.1 FCA-induced arthritis in the rat.

In the current investigation, a single local subdermal injection of low dose FCA around the rat ankle joint induced an arthritis that remained confined to the injected joint. The limited pathology associated with monoarthritis has considerable advantages over the widespread and systemic nature of adjuvant-induced polyarthritis because any observed changes (e.g. in mechanonociceptor discharge or behavioural parameters) are more likely to be directly attributable to the inflammatory process itself and not a consequence of severe pain and stress.

The mechanisms (e.g. immunological, biochemical) underlying the various phases of FCA-induced monoarthritis (see Section 3) have not been investigated in the present work. Thus, studies are required to correlate the various phases with, for example, changes in the involvement of macrophages, neutrophils, T-cells and serum albumin

or acute phase proteins levels (see Burnstein & Wakeman, 1964; Billingham, 1983; Schaible & Grubb, 1993).

The advantage of locally injecting FCA in the rat is that the extent of inflammation induced is simply determined by the dose of FCA injected. For example, as in the present experiments, a low dose of FCA (150μg) induced a monoarthritis, whereas other investigators, by increasing the dose of FCA (250μg), can obtain more severe forms such as bilateral arthritis (Donaldson et al., 1993). It would be of interest to perform studies which correlate articular C-fibre discharge or changes in behavioural parameters with inflammation induced by varying doses of FCA.

## 10.2 Articular mechanonociceptor discharge from normal and monoarthritic joints

A particular difficulty in the current neuropharmacological experiments was that it was not possible to record from the same afferent unit before and after induction of chronic FCA-induced arthritis. As a result, analysis of neural data is reduced to the less satisfactory procedure of comparing samples of mechanonociceptor activity from normal and arthritic joints. It has been shown that the same initially mechanoinsensitive afferent units in normal cat knee joints (identified by electrical stimulation of axons) become responsive to mechanical stimuli following acute inflammation of the joint induced by kaolin / carrageenan (Grigg et al., 1986 and Schaible & Schmidt 1988). Such receptors have been termed 'silent or sleeping nociceptors' or, more appropriately by Iggo (1988), 'inflammation receptors'. In view

of the findings of Grigg et al. (1986) and Schaible & Schmidt (1988), it would be of value to perform similar studies where neural activity is recorded from the same afferent unit before and after induction of acute arthritis in the rat ankle joint so as to determine the role of 'inflammation' receptors in this preparation.

In the present experiments, it was only possible to record action potentials in the nerve trunk by means of extracellular electrodes located several millimetres (50-150) from the nerve terminals. It is not possible to determine from such recordings the mechanisms responsible for action potential generation, nor to study the effects of drugs directly on the terminals of C-fibres. Thus, drug effects could be primary, on the nerve terminals, or secondary to actions on other cells (e.g. mast cells and macrophages), nerves (sympathetic neurones) or blood vessels. It would be of immense value to record changes in membrane potential from the terminals of C-fibres by using the patch-clamp technique. Unfortunately, it is not possible to make such recordings with the currently available technology. However, it may soon be possible to patch onto the terminal endings that sprout following a nerve resection (neuroma). Even if this proves possible, it will then be debatable whether the results obtained from such neuromas are relevant to the terminals of normal C-fibre afferents.

It has been shown that primary afferents are involved in 'cross over' (inflammation in the uninjected contralateral limb following inflammation in ipsilateral limb) of FCA-induced arthritis (Donaldson et al., 1993). In view of this finding, it would be interesting to perform studies which record alterations in C-fibre neural discharge in

the contralateral (untreated) joint following inflammation (FCA-induced arthritis), or electrical stimulation of C-fibres, in the ipsilateral joint.

The present experiments investigated neural activity from only high threshold slowly adapting capsaicin-sensitive C-fibres but did not study in detail neural discharge from other fibre types such as the low mechanical threshold-rapidly adapting fibres (i.e. those responding to gentle pulling of the superficial layers of articular connective tissue). Thus, it would be of value to record neural activity from these fibres in order to determine in detail whether their sensitivities are altered in inflamed joints. Other investigators have shown that fibres, other than C-afferents, such as the Aβ-fibres (large myelinated primary sensory neurones) show increased sensitivity following chronic inflammation, such that innocuous stimuli (e.g. touch) cause pain (Woolf & Doubell, 1994).

Neural recordings from C-fibres showed that articular mechanonociceptor discharge was elevated in arthritic joints as compared to untreated joints. How such elevations in neural discharge affect the excitability of neurones in the central nervous system (CNS) was not addressed in the current investigation. However, there is a large literature which describes the many changes in the CNS such as the 'wind-up' phenomenon or changes in the concentration of a large number of various transmitters and mediators (see Section 1.4.4). Alterations can also occur outwith the CNS. For example, use of molecular biological techniques have shown that neuropeptide expression (substance P and calcitonin gene-related peptide) is increased in the dorsal root ganglia of adjuvant-arthritic rats (Donaldson et al., 1992; Smith et al., 1992).

Increases in neural discharge from mechanonociceptors in arthritic joints can also contribute to joint inflammation by generating axon-reflexes in the terminal arborisations of C-fibres which gives rise to the release of various inflammatory neuropeptides including substance P and CGRP (see Section 3.4.3 and Foreman,1987). These C-fibre induced alterations can be avoided by early pharmacological intervention which reduces discharge from these afferents. However, in some cases of chronic pain and inflammation, alterations in the CNS can persist even without a C-fibre input (see Dray et al., 1994).

## 10.3 Neuropharmacological versus functional studies using FCA-induced monoarthritis

In addition to recording mechanonociceptor discharge from arthritic ankle joints in anaesthetised animals, functional studies were also performed using conscious monoarthritic rats. Acute recordings from C-fibres have the advantage that drug effects are determined in the short-term (seconds - minutes), whereas behavioural studies allow drug effects to be studied in the longer term and during chronic dosing. It seems reasonable that the two types of studies involve different measures of the same events. Thus, for example, the reduction in threshold for mechanical activation of mechanonociceptors in arthritic joints (neuropharmacological studies) corresponds to the reduced latency and increased sensitivity in paw withdrawal following application of mechanical pressure to the arthritic ankle joint (behavioural studies). Future investigations could clarify the relationship between behavioural changes and

C-fibre discharge from the same animal at fixed times (post-FCA injection), with and without treatment with drugs.

## 10.4 Effects of indomethacin

The results of the present experiments have shown that indomethacin not only reduced (but did not abolish) the elevated C-fibre discharge from mechanonociceptors, but also reduced (but did not abolish) the joint swelling, inflammation and mechanical hyperalgesia associated with monoarthritis (see Section 3). These results strongly suggest an important involvement of prostanoids in both the neuropharmacological and functional studies, although this does not exclude the additional involvement of other mediators (see Section 4.4.1 & 10.6).

Activation of cyclo-oxygenase (COX) enzymes catalyse the formation of prostanoids. It is now well known that there are two types of COX enzyme, termed COX-1 and COX-2. Of these, COX-1 is constitutive and active in normal conditions, whereas COX-2 is induced under inflammatory conditions, and the prostanoids formed may have pathological functions (Xie et al., 1991; Lee et al., 1992). Since increased levels of COX-2 have been reported in patients with rheumatoid arthritis and in adjuvant-arthritic rats (Sano et al., 1992), it would be of considerable interest to determine what role COX-2 has in adjuvant-induced monoarthritis. In order to investigate this, neuropharmacological and behavioural studies could be performed using NS-398 (N-[2-cyclohexyloxy-4-nitrophenyl]methanesulfonamide), a COX inhibitor with reported selectivity for the COX-2 form of the enzyme (Arai et al.,

1993; Futaki et al., 1994), and the results compared with those obtained with the non-selective COX inhibitor, indomethacin.

## 10.5 Role of purinoceptors, β-adrenoceptors and bradykinin receptors in FCA-induced arthritis

The current results showed that neither purinoceptors, whether $P_1$ (adenosine receptors) or $P_2$ (ATP receptors), nor β-adrenoceptors appear to be involved in modulating neural discharge recorded from mechanonociceptors in either normal or adjuvant-arthritic rat ankle joints. It has previously been shown that although $PGE_1$ or $PGE_2$ cause little or no increase in afferent discharge, they do potentiate the excitatory action of bradykinin (Chahl & Iggo, 1977; Birrell et al., 1993). Therefore, future studies should examine the possibility that purines or drugs acting at β-adrenoceptors could sensitise articular sensory receptors to inflammatory agents such as certain prostanoids, bradykinin or 5-HT.

It may be the case that purines or drugs selective for β-adrenoceptors affect neural excitability not at the periphery, but centrally. This could be investigated by recording from neurones in the CNS (e.g. spinal dorsal horn, thalamic or cortical neurones) whilst activating nociceptors in normal and arthritic joints. Another possibility is that purinoceptors and β-adrenoceptors are involved only in modulating afferent neural activity in the acute phase of inflammation or in inflammation induced by other agents which differs from FCA (e.g. in immune components or time course). Thus, it would be worthwhile performing neuropharmacological studies to assess the role of

purinoceptors and β-adrenoceptors using other models of inflammation (e.g. acutely induced by FCA, carrageenan, kaolin, urate crystals or latex spheres).

Behavioural studies using the adjuvant-induced monoarthritic rat model are required to determine what effects, if any, drugs affecting purinoceptors have on the inflammation and hyperalgesia associated with this preparation. These results could then be related to the current electrophysiological results to ascertain if there are any differences. Regarding β-adrenoceptors, the present behavioural studies showed, in agreement with the neuropharmacological investigations, that neither selective β-adrenoceptor agonists nor antagonists have any effect on FCA-induced monoarthritis.

Although both adrenaline and noradrenaline failed to affect neural discharge from mechanonociceptors in normal joints, they did enhance both spontaneous and mechanically-evoked activity in approximately 50% of C-fibre units studied in chronically-inflamed (adjuvant-arthritic) joints. The question of why these catecholamines did not affect neural activity recorded from some C-fibres in inflamed joints is not clear, but it may simply be due to some C-fibres not expressing or containing functional adrenoceptors, although why this should be so requires study - it may be the case that it is only a specific subpopulation of C-fibres which has the ability to respond to catecholamines following chronic inflammation. Since the effects of adrenaline or noradrenaline were not modified by propranolol, this strongly suggests that these catecholamines are not acting at β-adrenoceptors, but possibly at α-adrenoceptors. The question of whether α-adrenoceptors ($\alpha_1$- and/or the $\alpha_2$-subtype) are indeed responsible for enhancing neural discharge evoked by adrenaline

or noradrenaline in arthritic joints was not addressed in the present work, and investigation would require the use of selective $\alpha_1$ and $\alpha_2$-adrenoceptor agonists and antagonists. In addition, future studies could also determine the effects that selective $\alpha_1$ or $\alpha_2$-adrenoceptor antagonists have on the elevated neural discharge from mechanonociceptors in adjuvant-arthritic joints.

Neuropharmacological experiments are required to establish the precise site(s) of action of adrenaline and noradrenaline. Possible locations are the terminals of C-fibre afferents and the sympathetic nervous system (see Section 6.4.2.1). To investigate this, articular C-fibre neural discharge could be recorded from arthritic rats that have been sympathectomised either surgically or chemically, by treatment with guanethidine.

A particularly interesting finding of the present work was that the selective bradykinin $B_2$ receptor antagonist, Hoe140, caused insurmountable antagonism of bradykinin-induced excitation, but surmountable antagonism of bradykinin-evoked sensitisation. From these results one can speculate that subtypes of the $B_2$ receptor may exist. Whether these differential effects are common to all bradykinin $B_2$ receptor antagonists or are peculiar to Hoe140 requires further neuropharmacological investigations using other potent and stable $B_2$ receptor antagonists such as D-Arg[Hyp$^3$-Thi$^5$-D-Tic$^7$-Tic$^8$]-bradykinin, Arg(Tos)$^1$-Hyp$^3$ -Thi$^5$-D-Tic$^7$-Oic$^8$-bradykinin, NPC17731 and NPC17761 (Farmer et al., 1991b; Lembeck et al., 1991; Corrêa & Calixto, 1993). The possible existence of $B_2$ receptor subtypes was supported by studies which investigated the actions of Hoe140 in the electrically-

stimulated rat vas deferens (see Section 9). The main reason for choosing the electrically-stimulated rat vas deferens preparation was not to use it as a replacement for investigating the actions of bradykinin and Hoe 140 on articular C-fibres, but as a preparation for investigating further the surmountable and insurmountable actions of Hoe140 (see Section 9). Studies in the vas deferens also suggested that mechanisms such as pseudo-irreversibility could account for the differential actions of Hoe140. The question of whether or not the bradykinin $B_2$ receptors responsible for BK-induced excitation and sensitisation of articular sensory C-fibres are indeed equivalent to the $B_2$ receptors located on sympathetic neurones (causing BK-induced neurogenic responses) and smooth muscle (causing BK-induced musculotropic responses) of the vas deferens remains to be established. In such studies it will be necessary to consider the similarities and differences between C-fibres and sympathetic neurones such as, for example, the fact that both are unmyelinated but that while C-fibres have a mainly afferent role, sympathetics have an efferent one.

Although the present results showed that agonists and antagonists selective for bradykinin $B_1$ receptors had no effect on articular neural discharge recorded from C-fibres in arthritic joints, it is possible that effects can be observed using other models of joint inflammation. Pilot recordings from a novel preparation in this laboratory, the medial articular nerve - knee joint preparation into which IL-1β is injected intra-articularly, have indicated that $B_1$ receptors may play a role in inflammation. In this model, des-Arg$^9$-bradykinin induced an excitation which was antagonised by the bradykinin $B_1$ receptor antagonist, des-Arg$^9$-Leu$^8$-bradykinin.

The present studies showed that bradykinin receptor antagonists ($B_1$ or $B_2$ selective) did not affect C-fibre neural discharge recorded from arthritic ankle joints. However, this does not mean that bradykinin receptor antagonists will be of no value in the treatment of joint inflammation, as it has been demonstrated that $B_1$ and/or $B_2$ receptor antagonists reduce the swelling, oedema or mechanical hyperalgesia (this hyperalgesia may be via other fibre types such as Aβ-fibres) associated with arthritis (Sharma, 1993; Davis & Perkins, 1994).

It remains to be established which second messengers are involved in bradykinin-induced enhancements of articular C-fibre neural discharge. Preliminary studies *in-vitro* using the electrically-stimulated rat vas deferens indicate the involvement of protein kinase C in the responses to bradykinin (see Section 9). Studies are also needed to examine the role of G-proteins in the bradykinin-induced responses. Such studies will need to be performed *in-vitro*, using neural preparations such as the electrically-stimulated vas deferens, or more appropriately, the isolated medial articular nerve - knee joint preparation.

### 10.6 Other inflammatory mediators

This thesis was concerned with the role of bradykinin, purines, and catecholamines (acting at β-adrenoceptors) in sensitising mechanonociceptors during chronic inflammation. The vast literature relating to inflammatory mediators makes it clear that there is an increasing number of putative inflammatory mediators /

transmitters with the potential for altering neural discharge recorded from articular C-fibre afferents. Some examples include: nitric oxide (from endothelial cells, macrophages and leukocytes), endothelin, cytokines (from macrophages), nerve growth factor (from fibroblasts and endothelium), platelet activating factor (from most inflammatory cells including platelets, macrophages, polymorphs and basophils), leukotrienes (see Section 4.4.1) and various neuropeptides (from mast cells, sympathetic neurones, and primary afferent neurones) such as substance P, secretoneurin, galanin, calcitonin gene-related peptide, dynorphin, enkephalin and neuropeptide Y (Foreman, 1987; Ferreira et al., 1989; Maggi, 1991; Rang et al., 1991; Dray & Bevan, 1993; Lewin & Mendell, 1993; Schaible & Grubb, 1993; Dray et al., 1994; Kelly et al., 1994). It is unlikely that any one mediator can be the sole 'pain' mediator, but instead it is more likely that inflammation involves the complex interactions between many mediators. Much further work is needed to characterise the actions and interactions of inflammatory mediators on the discharge of articular sensory afferent nerves. Once a detailed understanding has been obtained concerning the effects of individual inflammatory mediators, particularly those responsible for the sensitisation of nociceptors during chronic inflammation, the knowledge gained will be valuable in the development of new potent analgesic and anti-inflammatory compounds for the treatment of chronic inflammatory conditions such as rheumatoid arthritis.

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# *APPENDIX I*

## *DETAILS AND SOURCES OF DRUGS USED*

**APPENDIX I**

**DETAILS AND SOURCES OF DRUGS USED**

| **Drug** | **vehicle for stock solution** | **Source** |
|---|---|---|
| **1)** 5`-N-ethylcarboxamidoadenosine (NECA) | saline (0.9% w/v) | Sigma |
| **2)** 8-phenyltheophylline (8-PT) | 99.95% saline (0.9% w/v); 0.05% 0.1M NaOH | Sigma |
| **3)** Adenosine | saline (0.9% w/v) | Sigma |
| **4)** Adrenaline | saline (0.9% w/v) | Sigma |
| **5)** α,β-methylene-ATP | saline (0.9% w/v) | Sigma |
| **6)** ATP | saline (0.9% w/v) | Sigma |
| **7)** Angiotensin II (human sequence) | saline (0.9% w/v) | NOVA biochem, UK |
| **8)** Atropine | saline (0.9% w/v) | Sigma |
| **9)** Bradykinin (BK), acetate salt | saline (0.9% w/v) | Sigma |
| **10)** Capsaicin (8-methyl-N-vanillyl-6-nonenamide) | 80% saline (0.9% w/v); 10% ethanol; 10% Tween 80 | Sigma |

| Drug | vehicle for stock solution | Source |
|---|---|---|
| **11)** Capsazepine (2-[-(4-chlorphenyl) ethylamino-thiocarbonyl]-7,8-dihydroxy-2,3,4,5-tetrahydro-1H-2-benzazepine) | 1% dimethylsulph-oxide | Cookson Biochemicals |
| **12)** DArg-Hyp$^{2}$-DPhe$^{7}$-BK | saline (0.9% w/v) | NOVA biochem, U.K. |
| **13)** DArg-Hyp$^{3}$-DPhe$^{7}$-BK (NPC567) | saline (0.9% w/v) | Bachem |
| **14)** DArg-Hyp$^{3}$-Thi$^{5,8}$-DPhe$^{7}$-BK (NPC349) | saline (0.9% w/v) | Bachem |
| **15)** DArg-Hyp$^{3}$-Thi$^{5}$-DTic$^{7}$-Oic$^{8}$-BK (Hoe140) | saline (0.9% w/v) | Glaxo, U.K. |
| **16)** des-Arg$^{9}$-BK | saline (0.9% w/v) | Sigma |
| **17)** des-Arg$^{9}$-Leu$^{8}$-BK | saline (0.9% w/v) | Sigma |
| **18)** DPhe$^{7}$-BK | saline (0.9% w/v) | Sigma |
| **19)** dexamethasone | saline (0.9% w/v) | Sigma |
| **20)** Freunds Complete Adjuvant (FCA) | heat killed *myco-bacterium tuberculosis* (1mg) suspended in mannide monooleate (0.15ml) and paraffin oil (0.85ml) | Sigma |

| Drug | vehicle for stock solution | Source |
|---|---|---|
| **21)** GR113808 | saline (0.9% w/v) | Glaxo, U.K. |
| **22)** GR82334 | saline (0.9% w/v) | Glaxo, U.K. |
| **23)** $Hyp^2$-DPhe$^7$-BK | saline (0.9% w/v) | NOVA biochem, UK |
| **24)** ICI 118551 (erthro-DL-1 (7-methyllindan-4-yloxy)-3-isopropylamino-butan-2-ol) | saline (0.9% w/v) | Sigma |
| **25)** Indomethacin | $NaHCO_3$ (5% w/v) | Sigma |
| **26)** Men10207 | saline (0.9% w/v) | Glaxo, U.K. |
| **27)** mepyramine | saline (0.9% w/v) | Glaxo, U.K. |
| **28)** Metrifudil | saline (0.9% w/v) | Glaxo, U.K. |
| **29)** L-noradrenaline bitartrate | saline (0.9% w/v) | Sigma |
| **30)** N-adamanteacetyl-DArg-$Hyp^3$-$Thi^{5,8}$-DPhe$^7$-BK | saline (0.9% w/v) | Sigma |
| **31)** $N^6$-cyclopentyladenosine (CPA) | saline (0.9% w/v) | Sigma |
| **32)** Ondansetron | saline (0.9% w/v) | Glaxo, U.K. |
| **33)** Prazosin | ethanol | Sigma |
| **34)** Propranolol | saline (0.9% w/v) | Sigma |
| **35)** Ranitidine | saline (0.9% w/v) | Glaxo, U.K. |
| **36)** Salbutamol | saline (0.9% w/v) | Sigma |

| Drug | vehicle for stock solution | Source |
|---|---|---|
| **37)** Salmeterol | 99.9% phosphate buffer pH 7; 0.1% glacial acetic acid | Glaxo, U.K. |
| **38)** Staurosporine | saline (0.9% w/v) | Sigma |
| **39)** Theophylline | 99.95% saline (0.9% w/v); 0.05% 0.1M NaOH | Sigma |
| **40)** $Thi^{5,8}$-DPhe$^{7}$-BK | saline (0.9% w/v) | Sigma |
| **41)** Thioperamide | saline (0.9% w/v) | Glaxo, U.K. |
| **42)** U46619 | saline (0.9% w/v) | Upjohn |

The chemicals for the physiological salt solution (Krebs) were of Analar grade and obtained from BDH Ltd. Dilutions of stock solutions were made in saline (0.9% w/v). The doses stated in the text are those of the salt where applicable.